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EDITORIAL

Is liver biopsy necessary in the management of alcoholic hepatitis?

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Abstract

Acute alcoholic hepatitis (AAH) is characterised by deep jaundice in patients with a history of heavy alcohol use, which can progress to liver failure. A clinical diagnosis of AAH can be challenging to make in patients without a clear alcohol history or in the presence of risk factors for other causes of acute liver failure. Other causes of acute on chronic liver failure such as sepsis or variceal haemorrhage should be considered. Liver biopsy remains the only reliable method to make an accurate diagnosis. However, there is controversy surrounding the use of liver biopsy in patients with AAH because of the risks of performing a percutaneous biopsy and limitations in access to transjugular biopsy. We review the existing literature and find there are few studies directly comparing clinical and histological diagnosis of AAH. In the small number of studies that have been conducted the correlation between a clinical and histological diagnosis of AAH is poor. Due to this lack of agreement together with difficulties in accessing transjugular liver biopsy outside tertiary referral centres and research institutions, we cannot advocate universal biopsy for AAH but there remains a definite role for liver biopsy where there is clinical diagnostic doubt or dual pathology. It also adds value in a clinical trial context to ensure a homogeneous trial population and to further our understanding of the disease pathology. Further prospective studies are required to determine whether non-invasive markers can be used to accurately diagnose AAH.

Key words: Alcoholic hepatitis; Liver biopsy; Diagnosis; Prognosis; Transjugular liver biopsy

Core tip: Acute alcoholic hepatitis (AAH) is a clinical syndrome of jaundice and coagulopathy in a patient with a recent history of heavy alcohol consumption. Clinical diagnosis is challenging and transjugular liver biopsy remains the gold standard. Here we discuss the literature which demonstrates there is a lack of agreement between clinical and histological diagnosis. This, together with limited availability of transjugular liver biopsy makes it impossible to advocate universal biopsy in all suspected cases of AAH. We suggest further research is conducted to prospectively compare histological and clinical parameters and to develop a reliable and accurate non-invasive diagnostic tool.

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INTRODUCTION

Acute alcoholic hepatitis (AAH) is a severe manifestation of alcoholic liver disease and is associated with a high short term mortality of 35% if untreated[1]. The clinical syndrome is characterised by a history of excessive alco-

hol consumption (> 80 g ethanol/d in males and > 60 g ethanol in females) and a recent onset of deep jaundice, which can lead to progressive liver failure. Symptoms are usually non-specific such as fatigue, weakness and anorexia but there is usually tender hepatomegaly and often fever, ascites and encephalopathy[2,3]. These features can also be seen in many hazardous drinkers making AAH challenging to diagnose clinically. Histology remains the gold standard in diagnosing AAH with well described features of steatosis, hepatocyte injury and neutrophil infiltration[4,5]. However, there are difficulties in access to transjugular liver biopsy and subsequent expert histopathology review limiting its utility.

Indeed, there is controversy over whether histology is essential in the diagnosis of AAH with the American Association for the Study of Liver Disease and European Association for the Study of the Liver offering different guidance[6,7]. Here, we discuss the existing evidence regarding liver biopsy in the management of AAH.

DIFFERENTIAL DIAGNOSIS OF AAH

Obtaining an accurate alcohol history is notoriously difficult but especially so in patients with potential AAH who are often unable to provide an accurate history due to symptoms of encephalopathy or acute alcohol withdrawal. Where there is uncertainty regarding recent heavy alcohol consumption as much evidence as possible should be obtained from relatives and friends or failing that primary or secondary care records. In all situations it is important to consider the differential diagnosis of acute liver failure including acute viral hepatitis, autoimmune hepatitis, Wilson's disease and drug induced liver injury. These factors can also co-exist in patients with heavy alcohol consumption, most commonly hepatitis C infection, which has been reported as a co-factor in up to 25% in one cohort[8]. Although clues to the diagnosis can be ascertained from the history and laboratory tests, in complex cases or where there is diagnostic doubt a liver biopsy often supplements the clinical information.

Studies which included histological diagnosis as an entry requirement have shown a variation in the prevalence of cirrhosis in patients with AAH from 65%-95%[9,10]. Therefore a minority of patients may present with AAH without features of chronic liver disease making it important to exclude other causes of acute liver failure.

However, in patients with chronic liver disease the key challenge in making a diagnosis of AAH is in differentiating it from other causes of acute on chronic liver failure (ACLF).

ACUTE ON CHRONIC LIVER FAILURE

ACLF is an increasingly recognised entity which, although not formally defined, has been the subject of 2 recent consensus meetings[11,12]. Both groups describe the condition as an acute deterioration in a patient with chronic liver disease associated with jaundice and coagulopathy[12] and high 3-mo mortality due to multiorgan failure[11]. In patients with alcohol-related cirrhosis AAH can be the precipitating cause of ACLF but other precipitants must be excluded especially gastrointestinal haemorrhage and sepsis of any source.

A recently published large multicentre prospective observational study was conducted to help establish diagnostic criteria for ACLF among European patients[13]. In 197 patients with alcohol-related liver disease (ALD) who met the criteria for ACLF, alcohol consumption within the preceding 3 mo was considered the precipitating event in 69 (35%) but because only small numbers underwent liver biopsy a histological diagnosis of alcoholic steatohepatitis (ASH) could not be made in these cases. The other commonest precipitants were bacterial infection and gastrointestinal haemorrhage.

Further information can be obtained from studies in patients with ACLF who underwent liver biopsy. In a series of 68 patients with acute decompensation of ALD 36 had a clinical diagnosis of AAH but only 18 of these (50%) had corresponding histological features of ASH, while a further 13 (19%) had histological ASH without clinical AAH[14]. In a separate study of 54 ALD patients admitted to hospital with ACLF a precipitating cause could only be identified in 30 (56%): 13 due to alcohol, 12 sepsis and 5 variceal bleeds[15].

These studies demonstrate that AAH is not always the cause of ACLF in patients with ALD; other causes of acute decompensation must be sought.

LIVER BIOPSY IN AAH PATIENTS

ASH, originally defined by an international consensus group, was described as the presence of steatosis, hepatocyte injury (ballooning and apoptosis) and polymorphonuclear infiltration[5]. Additional features of Mallory-Denk bodies and intraparenchymal cholestasis are observed but not necessary for diagnosis[16]. However, these characteristic changes of ASH can be seen in patients with ALD without the clinical syndrome of AAH or even active alcohol consumption. As described above 13 out of 68 (19%) patients with acute decompensation of ALD had histological ASH without corresponding clinical AAH[14]. ASH has also been noted in explant tissue from patients transplanted for ALD who were presumed to be abstinent. In 1 study ASH was noted in 32 of 148 (22%) explants from ALD patients including 25 who declared abstinence from alcohol for more than 6 mo[17]. A Spanish group reported 36 out of 68 (53%) explants from ALD patients had ASH, which was not associated with a reduced survival[18]. Therefore, it is important to be clear about terminology: we recommend the use of ASH to apply to the histological diagnosis while AAH should refer to the clinical syndrome.

In patients with severe AAH, many with deranged coagulation and significant ascites, the risks of performing a percutaneous liver biopsy are increased and a transjugular route is required. This is a well described and safe to per-

form procedure[19] which should be considered standard practice in hepatology centres[20]. In a systematic review of over 7500 transjugular liver biopsies minor bleeding (not requiring blood transfusion) and major complications were similar to the percutaneous approach (6.5% and 0.6% respectively) and death was rare at 0.09%[21]. No specific subgroup analysis was performed in those with coagulopathy as the indication but in 183 patients with congenital coagulopathy there was no mortality and the minor and major haemorrhagic complication rates were similar to the whole group (6% and 0.5% respectively). Sufficient biopsy material allowed a histological diagnosis to be made in 96.1% of samples with a median number of 2.7 passes[21]. In 132 patients presenting with AAH transjugular biopsy allowed accurate histological interpretation in 100% of cases with a mean length of 19 mm of tissue[22].

Unfortunately little attention has been paid to the timing of biopsy in AAH, which is usually unreported in clinical trials. Early biopsy, as is the practice of several liver centres (median time of 3 d in 1 centre)[22], may be more sensitive in confirming the diagnosis of AAH. Further studies are required to establish the optimal timing of liver biopsy.

Interpreting a liver biopsy specimen requires appropriate expertise and experience with access to specialist histopathologists but there still remains interobserver error. In patients with severe AAH and background cirrhosis this error has been shown to be minimal in one study with a high degree of concordance between 2 histopathologists ($\kappa = 0.77$)[23]. However, this was based in a specialist hepatology centre with expert liver pathologists and was lower in patients without cirrhosis ($\kappa = 0.65$).

Access to transjugular liver biopsy is variable and generally only available in tertiary referral centres and academic institutions. Transferring patients between centres only to obtain a liver biopsy is logistically challenging, may increase the risk to the patient and is associated with additional costs.

HISTOLOGICAL SCORES TO DETERMINE PROGNOSIS

There is evidence that some of the histological characteristics as well as liver expressed soluble factors, such as chemokines and interleukins, can be used to predict clinical outcome from AAH. This could assist clinical decision making and guide treatment choices. Steatosis < 20% is an independent predictor of poor outcome[24] and polymorphonuclear cell infiltrate is associated with severity of AAH[25] and is correlated with 1 year survival[10]. Liver tissue interleukin-8, a potent neutrophil chemoattractant, correlates with neutrophil infiltration and biochemical markers of outcome[26,27]. Intercellular adhesion molecule-1, a leukocyte adhesion molecule associated with T helper cell recruitment to sites of inflammation, is elevated in AAH versus fatty liver and its level correlates with histological hepatocellular damage[28]. CXC family chemokine expression correlates with prognosis[25] and CCL2 is elevated in AAH[29]. However, a histological scoring system combining these multiple parameters has not been developed and many of these individual predictors have not been validated nor are they routinely used outside a research setting. A histological severity score including K8/18 staining (a marker for hepatocyte ballooning) shows good accuracy for predicting 90-d survival but has not yet been validated in a second cohort[14]. Only 1 validated AAH histology score has been published in abstract form, finding that fibrosis stage, polymorphonuclear infiltrate, cholestasis and the presence of megamitochondria predicted 90 d mortality[30]. Further studies in this area are required to establish a reliable and reproducible histological scoring system that predicts clinical outcome.

USING CLINICAL SCORES TO DIAGNOSE AAH

An accurate non-invasive clinical test for AAH remains elusive. There are a host of different clinical scoring systems in the literature which have been developed to determine severity and likely benefit from glucocorticoid therapy (modified discriminant function[31]), prognosis (Glasgow Alcoholic Hepatitis Score[32]) or response to glucocorticoids (Lille score[33]). Little work has been conducted to examine how accurate these clinical scores are by comparing them to histological data. An abstract describing a literature review of 39 randomized controlled trials (RCTs) in AAH (11 of which had histological ASH as an entry criteria) suggested that overall 84.5% had histological ASH but this could be enriched to 96% if a minimum bilirubin level of 80 μmol/L was used[34]. Another study (also published only as an abstract) found that 70% of patients with a clinical diagnosis of AAH with an MDF ≥ 32 had histological confirmation of ASH[35]. Further work to prospectively compare clinical and histological diagnosis of AAH is needed and to establish whether non-invasive methods of diagnosing AAH can be used accurately. One such study is currently underway in a United Kingdom RCT (STOPAH; ISRCTN reference number: ISRCTN88782125), which intends to compare histological and clinical parameters as part of a secondary analysis. This pragmatic study, which aims to reflect everyday United Kingdom clinical practice, includes all patients with a clinical diagnosis of severe AAH (defined as recent onset jaundice with a bilirubin > 80 μmol/L and heavy alcohol consumption within the last 2 mo of > 80 g/d in men and > 60 g/d in women with a discriminant function ≥ 32) without the requirement for a biopsy. Liver histology from the subgroup that has a biopsy as part of an institution's standard clinical care will be compared to clinical parameters using this robust clinical definition of AAH.

CONCLUSION

Whilst it is clear that liver biopsy remains the recommended gold standard to diagnose ASH[6] and has the

potential to be used as a prognostic indicator, opinion remains divided on its practical clinical utility. Liver biopsy in this group of patients is as safe to obtain by the transjugular route and provides sufficient tissue to make a histological diagnosis compared to the percutaneous route. It is mandatory where there is diagnostic uncertainty and is useful where cirrhosis is not clinically suspected. However, the widespread clinical utility of liver biopsy is limited by the lack of provision of transjugular liver biopsy in non-specialist centres and cannot therefore be recommended to form part of routine practice outside these centres. In addition, the timely specialist interpretation that is essential to make the diagnosis of ASH is also generally restricted to specialist centres. Efforts need to be made to improve clinical diagnostic accuracy and studies are required to prospectively compare histological and clinical features in patients with AAH. There is the suggestion that accuracy can be improved by using a high cut-off of bilirubin > 80 μmol/L[34] but this also requires prospective validation, which may be provided by the STOPAH clinical trial.

Liver biopsy in AAH plays an important role in a research context: it can improve the homogeneity of the study population and reduce the risk of type Ⅱ error. It allows researchers to study the mechanisms of alcohol mediated liver damage and identify new targets for therapy. However, including only patients with histological ASH may not actually reflect the patient population we treat in everyday clinical practice but only a highly selected subgroup. Until a reliable non-invasive diagnostic method for the clinical syndrome of AAH has been developed and validated, clinical trials should continue to include patients defined by a robust clinical definition of AAH.

In summary, we recommend the clinical use of transjugular liver biopsy in patients with severe AAH only where there is irresolvable diagnostic uncertainty and as a research tool to further our understanding of the mechanisms and pathology of the disease.

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SCIENTOMETRICS

Improvement analysis of article quality in *World Journal of Gastroenterology* during 2008-2012

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Abstract

AIM: To understand the changes and development of *World Journal of Gastroenterology* (*WJG*) in recent years.

METHODS: The Journal Citation Report (JCR) and SCI-E database of the ISI Web of Knowledge were used to search the articles and data of related indices in *WJG* during 2008-2012. Bibliometric methods were used for statistical analysis of the author's degree of collaboration, collaboration rate, the first author's publications, high-productivity authors, the authors' origins in each year; the distribution of the countries and journals of the authors citing *WJG* papers was also analyzed. In addition, the indices related to this journal in each year were compared with the data from 6 SCI journals in the field of gastroenterology in the 2012 volume.

RESULTS: A total of 4409 papers in *WJG* were examined in this study. For the period 2008-2012, the self-citation rate was 8.59%, 6.02%, 5.50%, 4.47% and 5.21%. Of a total of 3898 first authors, 3526 published 1 paper, 291 published 2 papers, 59 published 3 papers, and 22 published 4 or more papers. The origin of *WJG* authors covered the six continents, and the majority came from Asia, Europe and North America. The number of countries of origin of *WJG* authors was 65, 66, 61, 65 an 60 for the period 2008-2012. Authors from 66 countries cited a total of 3194 of the 4409 papers, and these citations were found in 1140 journals.

CONCLUSION: The results suggest that *WJG* has stayed on the track of normal international publication and all the indices of this journal are stable and reasonable.

Key words: Author analysis; Bibliometrics; *World Journal of Gastroenterology*; Science Citation Index

Core tip: A total of 4409 articles were examined to explore the development of *World Journal of Gastroenterology* (*WJG*) during 2008-2012. Based on analysis of the relevant indices, this study not only discussed the development and changes of *WJG* in recent years, but also the characteristics of the published papers and the authors' origins. Furthermore, we performed analyses involving several journals of gastroenterology. The results show that all the indexes of this journal are stable and reasonable, and *WJG* has developed into one of the important journals in the field of gastroenterology.

Yang H, Chen YX. Improvement analysis of article quality in *World Journal of Gastroenterology* during 2008-2012. *World J Gastroenterol* 2013; 19(44): 7830-7835 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/7830.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.7830

INTRODUCTION

Journal quality evaluation is an important subject of concern to both editors and readers. Although the evaluation indexes are frequently the citation data of the papers

in a given journal such as the total citation frequency, impact factor and so on, paper publication data for the journal, such as the number of papers and authors' origins can also reflect the journal's academic status in the relevant disciplines. *World Journal of Gastroenterology* (*WJG*) is an English journal founded in 1995 and published by Baishideng Publishing Group. In 2005 and 2008, 2 papers analyzed the multiple indexes of *WJG* for the periods of 1998-2004 and 2001-2007, respectively[1,2]. Following the above 2 papers, this study compared and analyzed the various indexes of the papers published in *WJG* in each year from 2008-2012 and the citations of these papers. We also selected 6 internationally renowned journals of gastroenterology including *American Journal of Gastroenterology*, *BMC Gastroenterology*, *Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology* and *Scandinavian Journal of Gastroenterology* for comparative analysis of the relevant indexes with *WJG*. Based on analysis of the above indexes, this study intended to determine not only the development and changes of *WJG* in the past several years, but also the characteristics of the published papers and the origins of the authors of this journal.

MATERIALS AND METHODS

The Journal Citation Report (JCR) of ISI Web of Knowledge[3] and SCI-E[4] database were employed. The JCR database was searched to identify the number of references, the number of self-citations, the self-citation rate, and other indicators in *WJG* during 2008-2012. The SCI-E database was retrieved to identify the papers included in *WJG* every single year from 2008 to 2012; in addition, the relevant items including Title, Author, Source, Document Type, Times Cited, and Addresses were analyzed. Bibliometric methods were utilized for statistical analysis of the author's degree of collaboration, the collaboration rate, the first author's productivity, high-production authors, the authors' geographic areas and/or country related to this journal in each year; the distribution of the countries and journals for the authors citing *WJG* papers was also analyzed. In the meantime, the 2012 issues of *American Journal of Gastroenterology*, *BMC Gastroenterology*, *Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology*, *Scandinavian Journal of Gastroenterology* and *WJG* were retrieved and compared. The comparative indexes included the number of annual publications, the author's degree of collaboration, the collaboration rate, the number of countries of origin for all the authors, the proportion of papers written by native authors, the impact factor in 2012, discipline ranking and self-citation rate. Meanwhile, comparative analysis with *WJG* was carried out to determine the relative performance of various indexes of *WJG*.

RESULTS

Basic situation of WJG in 2008-2012

WJG published 48 issues yearly in 2008-2012, and during the period SCI-E indexed 1200, 964, 916, 762 and 1008 *WJG* items in the respective years, giving a total inclusion of 4850 items; the included 5 types of items were article, review, editorial, letter and biography. The number of indexed articles and reviews was 1112, 863, 813, 677 and 944 in the respective years, a total of 4409 papers. The results and conclusion of our research are from the analysis of these 4409 papers. Table 1 lists the number of references, the average number of references in each paper, the number of self-citations in the journal, the average number of self-citations and the self-citation rate.

Description of the authors of WJG papers between 2008 and 2012

There were 26600 authors from 4409 papers. Table 2 lists the distribution of the number of co-authors (mono-authorship and co-authorship), and 3898 were first authors; 3526 (90.46% of 3898 first authors) published 1 paper, 291 (7.47%) 2 papers, 59 (1.51%) 3 papers, 11 (0.28%) 4 papers, and 11 (0.28%) 5 or more papers. Table 3 shows the authors who published 5 or more papers.

Distribution of author's geographic area and main country

According to the 6 continents geographically, the authors' addresses were mainly located in Asia, Europe and North America (Figure 1). Table 4 lists the number of papers published by authors of the top 15 countries. Of the top 15 countries, there were 5 countries in Asia, 7 in Europe, 2 in North America and 1 in South America.

Distribution of the countries and journals for authors citing WJG papers

Authors from 66 countries cited 3194 of the papers (72.44%), with a total of 19872 citations. The authors from the United States of America were the top and responsible for 4716 citations (23.73% of the total); authors from China ranked second and were responsible for 3088 citations (15.54%); the third was Japan with 1617 citations (8.14%). The top 15 countries were responsible for 18889 citations (95.06% of the total cited) (Figure 2). These citations were from 2083 journals, and the top 15 of these journals gave 3373 citations (16.97% of the total) (Table 5).

Comparison of the relevant data of gastroenterological journals

The data of the JCR database can be used to analyze the citation status of journals, we can evaluate the quality of the journals in each discipline. The Gastroenterology and Hepatology category of JCR 2012 Science Edition included 74 journals, and mean value of impact factor of these journals was 3.115. The 7 representative journals are *American Journal of Gastroenterology*, *Gastroenterology*, *BMC Gastroenterology*, *Journal of Clinical Gastroenterology*, *Journal of Gastroenterology*, *Scandinavian Journal of Gastroenterology*, and *WJG*; of these, 3 journals are from North America, 2 from Europe, and 2 from Asia. Table 6 lists the number of papers, author's degree of collaboration,

Table 1 Literature indexes for papers published in *World Journal of Gastroenterology* between 2008 and 2012

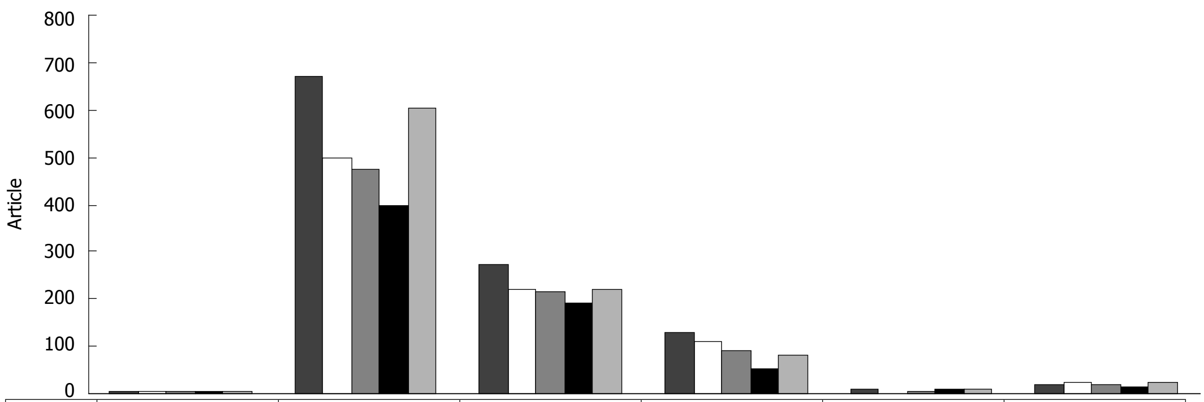
Year	No. of papers	No. of references	Average No. of references	No. of self-citations in the journal	The mean No. of self-citations in each paper	Self-citation rate
2008	1112	40485	36.41	930	0.84	8.59%
2009	863	29458	34.13	767	0.89	6.02%
2010	813	29624	36.44	832	1.02	5.50%
2011	677	25878	38.22	758	1.12	4.47%
2012	944	37947	40.20	918	0.97	5.21%

Table 2 Co-author collaboration status in *World Journal of Gastroenterology* in 2008-2012

Year	Distribution of number of co-author articles											Total (articles)	Authors	Cooperation degree	Cooperation rate
	1	2	3	4	5	6	7	8	9	10	≥ 11				
2008	49	96	91	147	154	167	111	121	63	42	71	1112	6501	5.85	95.59%
2009	28	66	100	102	139	107	107	74	46	38	56	863	5072	5.88	96.76%
2010	28	62	77	84	113	108	108	74	53	46	106	813	5037	6.20	96.56%
2011	21	46	63	77	103	87	100	96	29	25	30	677	4034	5.96	96.90%
2012	31	67	86	122	95	137	111	91	57	57	90	944	5956	6.31	96.72%
Total	157	337	417	532	604	606	537	456	248	208	353	4409	26600	6.03	96.44%

Table 3 Authors with 5 or more publications in *World Journal of Gastroenterology* between 2008 and 2012

Author	Institute	No. of papers of first authors	No. of papers of the communicating authors	No. of cited papers	Citation frequency
Freeman, Hugh James	Univ British Columbia Hosp, Canada	22	22	22	133
Tarantino, Giovanni	Univ Naples Federico II, Med Sch Naples, Italy	7	9	7	42
Ishikawa, Toru	Saiseikai Niigata Daini Hosp, Japan	7	7	5	20
Akbulut, Sami	Diyarbakir Educ and Res Hosp, Turkey	7	8	5	18
Hirasaki, Shoji	Sumitomo Besshi Hosp,and Kubo Hosp, Japan	7	8	6	27
Sporea, Ioan	Univ Med and Farm Timisoara, Romania	5	5	5	52
Katsinelos, Panagiotis	Cent Hosp, Greece	5	5	4	19
Lohsiriwat, Varut	Mahidol Univ, Siriraj Hosp, Thailand	5	5	5	48
Terada, Tadashi	Shizuoka City Shimizu Hosp, Japan	5	5	3	41
Sun, Long	Xiamen Univ, Affiliated Hosp 1, China	5	0	4	37
Lee, Tae Hoon	Soon Chun Hyang Univ, Coll Med, Cheonan Hosp, South Korea	5	2	4	9



	Africa	Asia	Europe	North America	Oceania	South America
2008	5	672	274	127	11	20
2009	5	500	219	110	1	26
2010	6	473	217	91	7	19
2011	7	398	193	55	10	13
2012	5	603	219	81	9	25

Figure 1 Distribution of *World Journal of Gastroenterology* authors among the continents between 2008 and 2012.

Table 4 Distribution of the top 15 countries or regions in *World Journal of Gastroenterology* during 2008-2012

Country name	2008	2009	2010	2011	2012	Total	Percentage
China	361	276	290	240	365	1532	34.75%
Japan	121	74	80	52	107	434	9.84%
United States	94	79	74	49	69	365	8.28%
South Korea	60	62	45	51	74	292	6.62%
Italy	54	53	53	60	56	276	6.26%
Germany	34	26	28	28	20	136	3.08%
Turkey	48	33	19	19	12	131	2.97%
Spain	31	35	13	16	21	116	2.63%
Greece	30	15	18	15	7	85	1.93%
England	23	18	16	9	17	83	1.88%
France	20	12	14	8	13	67	1.52%
Brazil	15	12	12	8	19	66	1.50%
Canada	23	13	9	3	11	59	1.34%
Netherlands	17	9	12	7	9	54	1.22%
Thailand	13	5	11	6	11	46	1.04%
Total	1305	998	984	811	1176	3742	84.87%

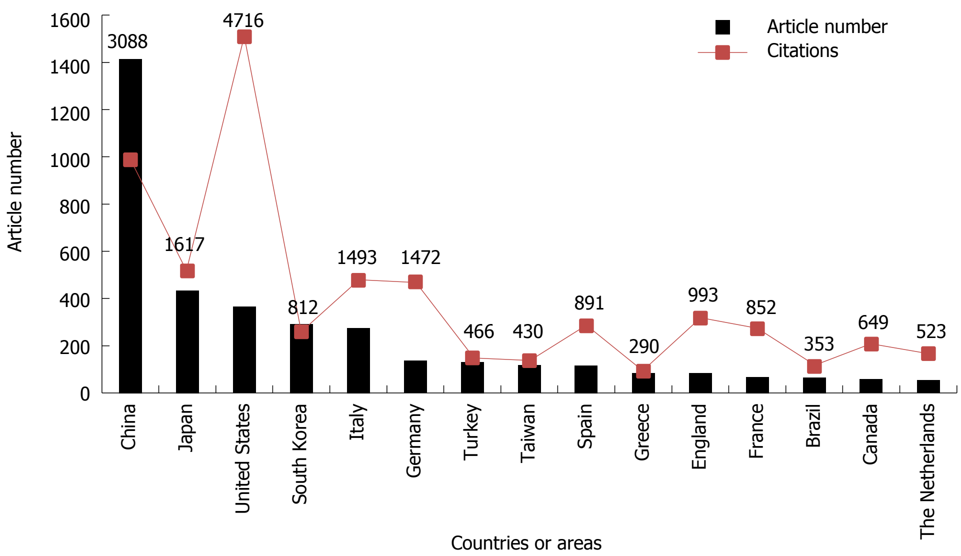


Figure 2 Comparison between the countries of authors citing *World Journal of Gastroenterology* papers of 2008-2012 and the countries of authors publishing *World Journal of Gastroenterology* papers.

Table 5 Main journals citing *World Journal of Gastroenterology* papers published between 2008 and 2012 *n* (%)

No.	Name of the citing journals	Quantity
1	*World J Gastroenterol*	1131 (5.69)
2	*PLoS One*	430 (2.16)
3	*Dig Dis Sci*	198 (1.00)
4	*Gastrointest Endosc*	180 (0.91)
5	*J Gastroenterol Hepatol*	171 (0.86)
6	*Aliment Pharmacol Ther*	147 (0.74)
7	*Inflamm Bowel Dis*	147 (0.74)
8	*J Hepatol*	138 (0.70)
9	*Hepatogastroenterology*	137 (0.69)
10	*Endoscopy*	122 (0.62)
11	*Eur J Gastroenterol Hepatol*	120 (0.60)
12	*Hepatology*	120 (0.60)
13	*Gastroenterology*	114 (0.57)
14	*Am J Gastroenterol*	110 (0.55)
15	*Scand J Gastroenterol*	108 (0.54)
	Total	3373 (16.97)

author's collaboration rate, geographical distribution of the authors, proportion of articles contributed by domestic authors, 2012 impact factor (IF), discipline ranking of the journal by IF, and self-citation rate for these 7 journals in 2012.

DISCUSSION

The publishing frequency of *WJG* was stable during 2008-2012, without significant changes in the annual number of papers published and the average number of papers in each issue. The average number of references in each paper increased gradually while the self-citation rate decreased gradually year by year. When compared with 2004, the average number of references in each paper in 2012 increased by 8.9[1], while the self-citation rate per article decreased by 0.73[1] in 2012; all the indexes were in a satisfactory state.

The degree of collaboration increased slightly while the

Table 6 Data comparisons of the 7 representative gastroenterology journals in 2012

Journal name	Articles published in 2012	Cooperation degree in 2012	Cooperation rate in 2012	Geographical distribution of authors	Ratio of articles contributed by domestic authors	2012 impact factor	Ranking of discipline impact factors	Self-citation rate in 2012
Am J Gastroenterol	190	7.50	94.74	32	United States 45.16	7.553	7	3.36
BMC Gastroenterol	165	7.35	98.79	36	China 21.21	2.11	42	2.6
Gastroenterology	278	10.55	97.48	44	United States 44.25	12.821	1	2.2
J Clin Gastroenterol	154	6.41	96.10	24	United States 38.96	3.203	23	3.1
J Gastroenterol	140	9.53	98.57	22	Japan 76.43	3.788	17	3.34
Scand J Gastroenterol	179	6.40	98.88	35	Sweden 21.79	2.156	40	3.29
World J Gastroenterol	944	6.31	96.72	58	China 35.67	2.547	34	5.21

collaboration rate decreased slightly during 2008-2012. The collaboration degree for each respective year was 5.85, 5.88, 6.20, 5.96 and 6.31; the mean collaboration degree was 6.03 and increased by 0.15 when compared with 5.88 during 2001-2007[2]. The collaboration rate for each year was 95.59%, 96.76%, 96.56%, 96.90% and 96.72%; the mean collaboration rate was 96.44%. In contrast with the slight increase in collaboration degree during 2008-2012, the collaboration rate during this period decreased by 1.22% when compared with the 97.66% during 2001-2007.

The origin of the authors diversified and the proportion of authors with 1 paper increased, but the high-productivity authors did not increase. During 2008-2012, 3526 authors published 1 paper in *WJG* accounting for 90.46% of the total authors, and 22 authors published 4 papers or more accounting for 0.56%. The core author group of this journal has yet to increase.

The number of author geographic areas increased: the origin of *WJG* authors became increasingly diversified; the authors came from 87 countries across the 6 continents of the world. Asia, Europe and North America were the main origins of the authors; and the proportion of authors from Asia was relatively stable; the number of papers contributed by authors from Asia was 672 (60.60%), 500 (58.07%), 473 (58.18%), 398 (58.88%) and 603 (63.88%) in respective years. The number of authors from Europe and North America changed little in each year and only decreased slightly in 2012. The number of papers contributed by authors from North America was slightly higher when compared with the data during 2006-2007[2] but the number was slightly lower during 2011-2012. The number of author countries exceeded 60 in each year; these authors came from a total of 87 countries and/or regions. The origin of the first authors of *WJG* became increasingly diversified and the number of originating countries increased when compared with the data of the period of 2001-2007.

The distribution of the author countries tended to be balanced: authors from the top 15 countries published 3742 (84.87%) papers. The proportion of papers published by Chinese authors showed an annual incremental trend, which coincided with an overall increase in the number of scientific publications in China. The ranking of the top 15 countries have changed; the contemporary top 5 countries were China, Japan, United States, South Korea and Italy. The international trend in the origin of *WJG* authors increased significantly.

During 2008-2012, 72.44% of all *WJG* papers were cited; although the time factor of 2012 may be responsible for the relatively lower number of citations, and therefore affected the citation rate of *WJG*, but the rate reflected the fairly satisfactory quality of *WJG* papers. The authors citing these papers were distributed among 66 countries or regions; American authors were ranked first and accounted for 23.73%, while Chinese authors were ranked second and accounted for 15.54%. The significant impact of *WJG* around the world was evidenced by the fact that 1140 journals cited *WJG* papers, and the distribution of the citing journals was dispersed widely.

The changes in *WJG* in the past 5 years were compared with another 6 journals of gastroenterology at the same time. *WJG* had the highest number of annual publications; authors' degree of collaboration in *WJG* was slightly lower than that of the other 6 journals, while the collaboration rate was within a reasonable range; the origin of *WJG* authors was the most diversified, and has gradually expanded from the predominant Chinese author group at the early stage to Asia and even the entire world across the 6 continents. Based on the data in JCR 2012, *WJG* ranked fifth of the 7 journals of gastroenterology; its self-citation rate declined and all other indexes were fairly reasonable.

In summary, *WJG* is attracting the attention of gastroenterologists globally, with authors scattered among 87 countries across the 6 continents. It has become a stage for gastroenterologists around the world to demonstrate their research findings. The author's geographic areas and countries are widely distributed, and all the indexes of this journal are stable and reasonable. *WJG* has embarked onto the track of normal internationalized publication, although it is still necessary to cultivate the core author group for the journal to establish its stable research characteristics.

COMMENTS

Background

The paper publication data of a journal can reflect a journal's academic status in the relevant disciplines, and the evaluation of journal quality is paid more and more attention by both editors and readers. In 2005 and 2008, two papers analyzed the multiple indices of *World Journal of Gastroenterology* (*WJG*) for the periods 1998-2004 and 2001-2007. Therefore, it was necessary to analyze the

relevant indexes and make an objective evaluation of journal quality in recent years.

Research frontiers

Although the evaluation indexes are frequently the citation data of the papers in a given journal such as the total citation frequency, impact factor and so on, the paper's publication data of the journal such as the number of papers and author's origin can also reflect the journal's academic status in the relevant disciplines.

Innovations and breakthroughs

Based on analysis of the relevant indexes, the authors only studied the development and changes of *WJG* during 2008-2012, but also the characteristics of the published papers and the origin of the authors of this journal.

Applications

The results indicate that *WJG* has become an international publication, and gastroenterologists around the world can report their research findings in this journal.

Terminology

The impact factor of a journal is a measure of the citations to science and social science journals, and is frequently used as a proxy for the importance of a journal to its field, with journals with higher impact factors deemed to be more important than those with lower ones.

Peer review

Commendable effort. Much needed analysis to establish the position of *WJG* in the field of gastroenterology and hepatology. In this interesting paper, authors performed analyses involving several journals of gastroenterology. All aspects of comparison are presented sufficiently. Statistical analysis of the study made clear and demonstrate that *WJG* has developed into one of the important journals in the field of gastroenterology.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Non-coding RNAs in hepatitis C-induced hepatocellular carcinoma: Dysregulation and implications for early detection, diagnosis and therapy

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Abstract

Hepatitis C virus (HCV) infection is one of main causes of hepatocellular carcinoma (HCC) and the prevalence of HCV-associated HCC is on the rise worldwide. It is particularly important and helpful to identify potential markers for screening and early diagnosis of HCC among high-risk individuals with chronic hepatitis C, and to identify target molecules for the prevention and treatment of HCV-associated-HCC. Small non-coding RNAs, mainly microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) with size greater than 200 nucleotides, are likely to play important roles in a variety of biological processes, including development and progression of HCC. For the most part their underlying mechanisms of action remain largely unknown. In recent years, with the advance of high-resolution of microarray and application of next generation sequencing techniques, a significant number of non-coding RNAs (ncRNAs) associated with HCC, particularly caused by HCV infection, have been found to be differentially expressed and to be involved in pathogenesis of HCV-associated HCC. In this review, we focus on recent studies of ncRNAs, especially miRNAs and lncRNAs related to HCV-induced HCC. We summarize those ncRNAs aberrantly expressed in HCV-associated HCC and highlight the potential uses of ncRNAs in early detection, diagnosis and therapy of HCV-associated HCC. We also discuss the limitations of recent studies, and suggest future directions for research in the field. miRNAs, lncRNAs and their target genes may represent new candidate molecules for the prevention, diagnosis and treatment of HCC in patients with HCV infection. Studies of the potential uses of miRNAs and lncRNAs as diagnostic tools or therapies are still in their infancy.

Key words: MicroRNA; Long non-coding RNAs; Non-coding RNAs; Hepatitis C virus; Hepatocellular carcinoma

Core tip: Regulatory non-coding RNAs (ncRNAs), mainly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are likely to play important roles in a variety of biological processes, including development and progression of hepatitis C-induced hepatocellular carcinoma (HCC). In this review, we focus on recent studies of ncRNAs, especially miRNAs and lncRNAs associated with hepatitis C virus (HCV)-induced HCC. We summarize those ncRNAs aberrantly expressed in HCV-induced HCC and highlight the potential of these ncRNAs to aid in early detection, diagnosis and therapy of HCV-induced HCC. Further, we discuss the limitations of current studies, and suggest future directions for research in the field.

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INTRODUCTION

Non-coding RNAs (ncRNAs) are transcribed RNA molecules with little or non-protein coding capacity; they represent approximately 97% of RNAs in higher eukaryotic organisms. ncRNAs include structural or housekeeping ncRNAs such as transfer RNA, ribosomal RNA, small nuclear RNA and small nucleolar RNA, as well as regulatory ncRNAs, which function to regulate gene expression. Based on transcript size, regulatory ncRNAs are classified into two major groups, small ncRNAs such as microRNAs (miRNAs), approximately 22 nucleotides (nt) in length, and long non-coding RNAs (lncRNAs) with sizes longer than 200 nt (Figure 1). Based upon a large number of experimental studies carried out over the past decades or two, it is now generally well-accepted that miRNAs play an important role in the regulation of gene expression primarily through post-transcriptional destabilization, translational repression of target mRNAs which bear complementary sites, or a combination of these two mechanisms[1-4]. With the development of next generation sequencing (NGS) techniques, a growing number of lncRNAs have been identified, characterized and functionally annotated[5,6]. lncRNAs are still among the least well-understood of transcripts. Several lines of evidence have suggested that lncRNAs are biologically functional rather than transcriptional "noise"[5,6]. Thus, lncRNAs have recently enjoyed increased and deserved attention, although the underlying mechanisms by which they function remain largely unexplored and unifying theories regarding their actions are still vague. ncRNAs including miRNAs and lncRNAs have been reported to be associated with cancer, including hepatocellular carcinoma (HCC), a highly prevalent and deadly cancer because of its frequent recurrence and/or metastasis.

HCC is among the most frequent forms of cancer worldwide, and its incidence is increasing rapidly. This increase is related to several factors. Chief among these are chronic hepatitis B and C (CHC) infections, and fatty liver disease. Indeed, hepatitis C virus (HCV) infection is one of the leading underlying causes of HCC, increasing the risk for HCC development by nearly 17-fold compared to healthy individuals[7,8]. In recent decades and especially in recent years, HCC incidence has increased sharply, and has been attributed largely to HCV infection. HCV-induced HCC typically develops in the setting of cirrhosis (advanced chronic liver diseases), although it does also occur in the absence of cirrhosis. Similarly, the development of HCC has been observed in mice expressing HCV transgenes in the absence of appreciable hepatic inflammation and fibrosis, suggesting that HCV infection is likely to have direct and unique cancer-promoting effects, which may be different from other carcinogenic factors such as those due to hepatitis B virus (HBV) and fatty liver disease. Understanding and insight into unique ncRNAs involved in HCV-induced HCC may suggest new approaches for diagnosis, prevention and treatment of HCV-induced HCC. To date, there have been few reports on differentially expressed lncRNAs in HCV-induced HCC. In this review, we will summarize recent studies regarding ncRNAs related to HCV-induced HCC. We will then address the potential utility of these ncRNAs in early detection, diagnosis and therapy of HCV-associated HCC. Finally, we will discuss the limitations of current knowledge, and suggest future directions for research in this field.

DYSREGULATED NCRNAS IN HCV-INDUCED HCC

microRNAs

miRNAs regulate gene expression primarily through post-transcriptional repression[3,4,9,10]. Sequence complementarity in the 6-8 base pair "seed regions" at the end of miRNA-mRNA heteroduplexes seem to determine the specificity of miRNA-target RNA interactions[11]. miRNAs are likely to play significant roles in the development and progression of cancers, including HCC[12,13] and HCV replication[9,14-16]. Identification and characterization of dysregulated miRNAs specific to HCV-induced HCC in tissue- and biofluid-based studies are important and helpful to reveal therapeutic targets or diagnostic markers, in particular, molecular signatures for the detection and early diagnosis of HCC among HCV patients in high-risk groups. The miRNAs reported differentially expressed in HCV-induced HCC are summarized in Tables 1 and 2, and we now summarize their known biological functions, and the molecular mechanisms and pathways in which they might be involved.

Up-regulated miRNAs

miRNAs profiling studies in HCV-induced HCCs compared with paired controls have found that a number of miRNAs are significantly elevated in HCV-induced HCCs, compared with normal controls. miR-1269 is the most increased in HCV-associated HCCs in contrast to normal livers, HCV-associated cirrhosis, or HBV-associated liver failure. Up-regulation of miR-1269 has also been found in other cancers such as breast cancer[17], colorectal cancer[18] and laryngeal squamous cell carcinoma (LSCC), one of the most common head and neck malignancies with no significant difference between tumors with and without lymphatic metastasis, suggesting that miR-1269 did not affect metastasis of LSCC. Thereto date there have been few, if any, reports on function and role of miR-1269 in HCC. Nevertheless, the increased expression of miR-1269 in HCV-induced HCC when compared with controls suggests that this miRNA may have an on-

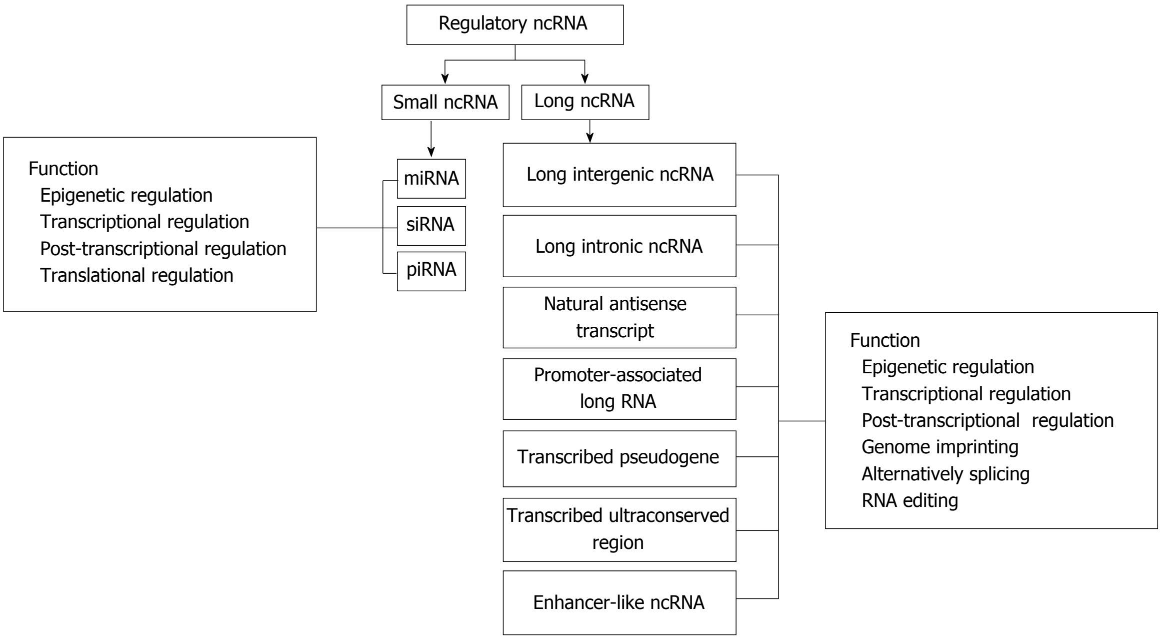

Figure 1 Classification of regulatory non-coding RNA and function in gene regulation. Regulatory non-coding RNA (ncRNAs) are divided into two major groups based on transcript size, small ncRNAs such as microRNA, small interfering RNA and piwi-interacting RNA (piRNA), as well as long ncRNAs with size greater than 200 nt. Both small ncRNA and long ncRNA have important regulatory function in gene expression. miRNA: microRNA; siRNA: small interfering RNA; piRNA: piwi-interacting RNA.

Table 1 Summary of microRNAs significantly up-regulated in hepatitis C virus-induced hepatocellular carcinoma

ncRNAs	Chromosomal location	Differential expression level	Clinical relevance	Ref.
Liver miRNAs				
miR-1269	4q13.2	15.7-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-224	Xq28	10.7-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-452	Xq28	10.1-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-224-3p	Xq28	8.1-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-221	Xp11.3	3.7-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-122	18q21.31	> 2-fold, HCV-associated HCC (n = 43) *vs* normal livers (n = 3), P < 0.01; HCV associated dysplastic nodules (n = 9) *vs* normal livers (n = 3), P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-100	11q24.1	> 2-fold, HCV-associated HCC (n = 43) *vs* normal livers (n = 3); HCV associated dysplastic nodules (n = 9) *vs* normal livers (n = 3)	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-10a	17q21.32	> 2-fold, HCV-associated HCC (n = 43) *vs* normal livers (n = 3); HCV associated dysplastic nodules (n = 9) *vs* normal livers (n = 3)	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
Urinary miRNAs				
miR-625	14q23.3	> 3-fold, HCV-associated HCC (n = 32) *vs* normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]
miR-532	Xp11.23	> 3-fold, HCV-associated HCC (n = 32) *vs* normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]
miR-618	12q21.31	> 3-fold, HCV-associated HCC (n = 32) *vs* normal urine samples (n = 12), P < 0.05	Potential marker for the detection/early diagnosis of HCC among high-risk HCV patients	[33]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; miRNAs: MicroRNAs; ncRNAs: Non-coding RNAs.

Table 2 Summary of microRNAs down-regulated in hepatitis C virus-induced hepatocellular carcinoma

ncRNAs	Chromosomal location	Differential expression level	Clinical relevance	References
Liver miRNAs				
miR-199a-5p	19q13.3	7.2-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-199a-3p	19q13.3	6.9-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-199b-3p	19q13.3	6.2-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-214	1q24.3	5.5-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-198	19p13.3	Approximately 5-fold, HCV-associated HCC (n = 43) *vs* normal livers (n = 3), P < 0.01; HCV-associated Dysplastic nodules (n = 9) *vs* normal livers (n = 3), P < 0.01	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
miR-139-3p	11q13.4	4.6-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-139-5p	11q13.4	4.4-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-424-3p	Xq26.3	3.9-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-125a-5p	19q13	3.7-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-130a	11q12.1	2.9-fold, HCV-associated HCC (n = 9) *vs* normal livers (n = 12) and other liver diseases [HCV-associated cirrhosis (n = 10), HBV-associated accurate liver failure (n = 4)], P < 0.05	Potential therapeutic target molecule to prevent HCV-induced HCC	[32]
miR-145	5q32	> 2-fold; HCV-associated HCC (n = 43) *vs* normal livers (n = 3); HCV associated dysplastic nodules (n = 9) *vs* normal livers (n = 3)	Potential therapeutic target molecule to prevent HCV-induced HCC	[30]
Urinary miRNAs				
miR-516-5p	19q13.42	> 3-fold, HCV-associated HCC (n = 32) *vs* normal urine samples (n = 12), P < 0.05	Potential marker for early diagnosis of HCC among high-risk HCV patients	[33]
miR-650	22q11.22	> 3-fold, HCV-associated HCC (n = 32) *vs* normal urine samples (n = 12), P < 0.05	Potential marker for early diagnosis of HCC among high-risk HCV patients	[33]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; miRNAs: MicroRNAs; ncRNAs: Non-coding RNAs.

cogenic role in HCV-induced HCC.

Interestingly, miR-224, miR-224-3p and their precursor are significantly up-regulated in HCV-associated HCCs compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure. miR-452, also significantly up-regulated in HCV-induced HCC, was recently shown to be coordinately expressed with its neighboring miR-224 in HCC through epigenetic mechanisms[19]. The DNA that encodes miR-224 is located on the X-Chromosome. miR-224 has been reported to be a cancer-related miRNA, including in HCC. Wang *et al*[20] identified and validated the apoptosis inhibitor 5 (API-5) as a specific target gene for miR-224. Additionally, SMAD family member 4 (*SMAD4*) has been identified as another target gene for miR-224. Over-expression of miR-224 increases the concentration of SMAD4 protein in murine granulosa cells, while SMAD4 RNA levels remain unchanged, suggesting a post-transcriptional role for miRNA-224[21]. It is likely that miR-224 plays a role in cell proliferation, migration, invasion, and anti-apoptosis in HCC, and is involved in hepatocarcinogenesis by directly binding to its validated gene targets such as *API-5*, *SMAD4*, *etc.*[20,22,23].

miR-122, a liver specific miRNA, is the most abundant miRNA expressed in hepatocytes (accounting for approximately 70% of total miRNA's) and the most extensively studied miRNA in liver diseases. miR-122 has major effects on several enzymes of cholesterol metabolism[24,25]. Unexpectedly, miR-122 was also shown to be required for HCV replication[14-16]. The effects of miR-122 depend upon the context and location of its cognate seed sequence binding sites. The sites in the 5' region are mostly associated with up-regulation of expression, whereas those in the 3' untranslated region are mostly associated with repression of expression[26]. miR-122 exerts several functions in the HCV life cycle[27,28]. Recent

studies have shown that miR-122 acts to protect HCV genome from degradation, and therefore stabilizes HCV RNA by decreasing activity of the cytosolic exonuclease Xrn1[27,28]. The role of miR-122 in HCC has been controversial. Some but not all studies suggest that miRNA-122 is preserved and increased specifically in HCV-associated HCC[12,29,30]. Nevertheless, a decrease in expression of miR-122 or undetectable miRNA-122 in human hepatoma cell lines such as HepG2 and Hep3B cell has been observed[31]. In parallel with these observations, overexpression of miR-122 inhibits anchorage-independent growth, migration, invasion and tumor formation in nude mice[31]. This needs to be further studied in the future.

Down-regulated miRNAs

The miR-199 family members including miR-199a-5p, miR-199a-3p and miR-199b are the most down-regulated miRNAs in HCV-induced HCC compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure[32]. miR-199a/b-3p is the third most highly expressed miRNA in the liver[34], and was also found to be consistently decreased in HCC patients with HBV infection[34] and alcohol consumption[35]. Its decrement significantly correlates with poor survival of HCC patients[34]. Down-regulation of miR-199a-3p results in a pronounced increase in cell proliferation while overexpression miR-199a-3p inhibits cell proliferation by imposing G_1 cell cycle arrest[36]. The target mRNAs for the miR-199a-3p have been predicted using bioinformatic approaches and validated experimentally. For example, mammalian target of rapamycin (mTOR) has been identified as one of important targets for miR-199a-3p binding. Through negative regulation of oncogenic mTOR, miR-199a-3p inhibits tumor proliferation[37].

miR-214 has been reported to be down-regulated by 5.5-fold in HCV-induced HCC compared to normal livers, HCV-associated cirrhosis and HBV-associated liver failure[32]. The down-regulation of miR-214 has been reported in HCC[20,38,39] and other cancers such as cervical cancer, whereas increase in miR-214 was found to significantly reduce growth of Hela cells[40]. In addition, reduced level of miR-214 is associated with invasion, stem-like traits and early recurrence of HCC[41]. Re-expression of miR-214 significantly suppressed the growth of HCC cells *in vitro* and reduced the tumorigenicity *in vivo*[41]. In the same study, the enhancer of zeste homologue (EZH2) and β-catenin (CTNNB1) were identified and validated as two functional target mRNAs of miR-214. Silencing miR-214 increased stem-like cells through activation of CTNNB1. Furthermore, the up-regulation of EZH2, CTNNB1 and the down-regulation of E-cadherin (CDH1), known to inhibit cell invasion and metastasis in HCC patients, correlated with earlier recurrent HCC and were independent predictors of poor survival.

LncRNAs

The discovery of lncRNAs ushered in a new and exciting area of study, although at the time lncRNAs were first found, they were considered to be merely transcriptional "noise"[5]. Recently, with fast development and application of NGS techniques, the numbers of lncRNAs continues to grow at a rapid pace, and it is increasingly clear that lncRNAs are a new class of regulators of gene expression, being involved in diverse biological processes and human diseases such as cancer. The association of lncRNAs with HCC has been studied and summarized[42], although the mechanisms whereby effects of the lncRNAs are realized are largely unknown. Analysis of the differentially expressed lncRNAs in HCCs (underlying etiology not specified) have revealed that a number of lncRNAs such as HOTAIR[43-46], HEIH[47], MVIH[48], MALAT-1)[49], HULC[50-52], H19[53-55], CUDR[56], YIYA[57], lncRNA-Dreh[58], lncRNA-LET[59] and MEG3[60,61] are associated with HCC. Most of these lncRNAs are up-regulated in HCCs, but less expressed or undetectable in normal controls. HCC patients with HOTAIR expression had significantly poorer prognoses and larger primary tumor sizes than those without HOTAIR expression[46]. Moreover, introduction of HOTAIR into human liver cancer cells promoted more rapid proliferation compared to controls[46]. Functional gene annotation analysis of TUC338 indicated predominant effect on genes involved in cell growth in both human and murine cells, suggesting that TUC338 plays a critical role in regulation of transformed cell growth and in the pathobiology of HCC [62]. Lai *et al*[49] found up-regulation of MALAT-1 in both liver cancer cell lines and HCC patient samples. HCC patients with high level of MALAT1 had a significantly increased risk of tumor recurrence after liver transplantation. MVIH was identified to be related to frequent microvascular invasion and higher tumor-node-metastasis stages as well as to decreased overall survival. In addition, in mouse models it promoted tumor growth and intrahepatic metastasis by activating angiogenesis[48]. The expression level of HEIH in HBV-induced HCCs is significantly associated with recurrence and is an important independent prognostic factor for survival[47]. Further studies indicated that HEIH plays a key role in the regulation of zeste homolog 2 (EZH2) and that this association was required for the repression of EZH2 target genes, suggesting that HEIH is an oncogenic lncRNA that promotes tumor progression[47]. Thus far, few studies have been focused on lncRNAs specific to HCV-induced HCC although HCV infection is one of the major causes of HCC, and HBV and HCV cause hepatocarcinogenesis by different mechanisms.

CLINICAL IMPLICATIONS FOR DIAGNOSIS AND THERAPY

miRNAs, lncRNAs and their target genes comprise a large and still growing number of candidate molecules for the prevention, diagnosis and treatment of HCC in patients with HCV infection, and studies of the potential use of miRNAs and lncRNAs as therapeutic or diag-

nostic approaches is still in its infancy. In this section, we mainly discuss clinical potentials of miRNAs and lncRNAs for HCV-induced HCC diagnosis and therapy.

miRNAs and lncRNAs to aid in diagnosis of HCV-induced HCC

The biomarkers currently available for screening and early diagnosis of HCC, including serum alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin, and AFP-L3 fraction or assaying cells from tissue biopsy by needle aspiration and surgical resection, suffer from numerous limitations[63,64]. Most patients chronically infected with HCV are asymptomatic for many years, and the average time to develop HCC after onset of HCV infection is about 28 years. The long latency period between initial HCV infection and development of HCC provides an important time window of opportunity for individuals to be monitored for disease progression and intervention. Therefore, the development of more reliable markers for diagnosis of HCC at an early stage and better approaches for HCC screening and early detection are urgently needed. The recent study from Abdalla *et al*[33] to identify urinary miRNAs as biomarkers specific for early detection of HCV-induced HCC, appears to be attractive and promising. The significantly up-regulated and down-regulated urinary miRNAs as listed in Tables 1 and 2 can be considered as promising candidate miRNA urinary markers for the early detection and diagnosis of HCV-induced HCC among high-risk HCV patients (Genotype 4). Of the identified miRNAs, miR-618 was found to have a sensitivity of 64% and a specificity of 68% for detecting HCC among HCV-positive individuals, whereas the sensitivity and specificity of urinary miR-650 were 72% and 58%, respectively. Also worthy of note, miR-618/650 in tandem improved the specificity to 75%, greater than the traditional methods based on serum levels of AFP. The urinary miRNAs signatures found in this study may be of great value and applied for the early diagnosis of HCC, before the onset of disease in high-risk patients infected with HCV. However, it is noted that this study was carried out in patients infected with HCV genotype 4, the most prevalent HCV genotype in Egypt. The potential for their use in the early diagnosis of HCC caused by different HCV genotypes other than genotype 4 needs further investigation and independent confirmation. So far, few studies have been reported regarding on lncRNAs signatures in biofluids specific to HCV-induced HCC, which may represent an exciting area for future exploration.

miRNAs and lncRNAs for HCV-induced HCC therapy

Recent studies have suggested the exciting possibility that ncRNAs may represent a novel therapeutic strategy for human diseases. The miR-122 antagonist, miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122 in a highly stable heteroduplex, already has shown promising results in phase 2a clinical trials at seven international sites. In this clinical study, Janssen *et al*[65] evaluated the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. This landmark study demonstrated that the use of miravirsen produced prolonged dose-dependent reductions in HCV RNA with no evidence of development of viral resistance. Meanwhile, targeting a number of other miRNAs such as miR-33a/b for the treatment of atherosclerosis[66,67], miR-208/449 for chronic heart failure, miR-34 and the let-7 miRNA for cancer, await entering clinical trials. In addition, Coelho *et al*[68] has recently demonstrated a new therapeutic approach to transthyretin amyloidosis by RNA interference (RNAi). In this phase I clinical trial, a potent antitransthyretin small interfering RNA was encapsulated in lipid nanoparticles, delivered to human hepatocytes, and resulted in a significant reduction of transthyretin, establishing proof of concept for RNAi therapy[68].

As summarized and discussed earlier in this review, many of the significantly dysregulated cellular miRNAs as listed in Tables 1 and 2, and currently found to be involved in the modulation of cell growth, apoptosis and invasiveness, can be considered as potential therapeutic targets for HCV-induced HCC therapy. The overexpression of these specific mature miRNAs can be achieved by synthetic miRNAs mimics or expression vectors. When inhibition of the selected miRNAs is desirable, antagomir or antisense oligos complementary to the specific miRNAs can be used. However, the introduction of the miRNA-based agents into clinical trials and the development of new therapeutic agents are hampered by a number of factors. The major road block is still the big challenge of developing a small animal model used in biomedical research to understand roles of ncRNAs in the pathogenesis of HCV-induced HCC. Among nonhuman species, only chimpanzees have thus far been capable of being infected with HCV, and disease in them is generally relatively mild. Most recently, Dorner *et al*[69] has reported a breakthrough and milestone in development of a genetically humanized mouse model for HCV research, in which the entire HCV life cycle can be completed and immune system is fully functioning. This genetically humanized mouse model will allow us to gain new insights into not only an important biology of HCV but also carcinogenesis of HCC caused by HCV. Additional challenging factors remain which slow the progress of the miRNA-based agents into clinical trials and new drugs. The dysregulated miRNAs in HCV-induced HCC as discussed earlier in this review were identified in individual studies, lacking consensus among the different reports, having been attributed to the differences among miRNA probe, staging and grade of malignancy of the tumor and different HCV genotypes. Therefore, there is still a long way to go before the miRNA-based therapy can be used in the clinic in the prevention and treatment of HCV-induced HCC.

CONCLUSION

As we summarized and discussed in this review, the re-

cent findings of roles of ncRNAs in not only regulating HCV life cycle but also their contribution to pathogenesis of HCV-induced HCC have been remarkable, despite the fact that we may have unveiled only a small portion of the very large number of ncRNAs; this is probably especially true for lncRNAs. We are only beginning to understand the nature and extent of the involvement of lncRNAs in disease. Recently, a number of lncRNAs have been found to be aberrantly regulated in cancer, including HCC[42-48,53-55,57,59-62]. However, at the present time there are no reports on aberrantly lncRNAs exclusively associated with HCV-induced HCC, and thus remain a large unexplored and undefined area, which may allow us to better understand the role of lncRNAs in the pathogenesis of HCV infections and HCC and to identify better therapeutic targets and more sensitive diagnostic markers.

The intracellular ncRNAs that were significantly altered in HCV-induced HCC have been proposed to be potential molecular targets for therapy to combat HCV and HCV-induced HCC. Furthermore, over the past years, extracellular ncRNAs, particularly ncRNAs in exosomes, have risen to be promising as biomarkers with diagnostic or prognostic value. Exosomes (30-100 nm in diameter) are one of the class of microvesicles found in biofluids, including blood, ascites fluid, urine, culture media of cell cultures, *etc.* Exosomes carry with them various nucleic acids, including ncRNAs (*e.g.*, miRNAs and lncRNAs) and proteins from their cells of origin, which allow us to achieve access to molecular information about their cell-of-origin without biopsying or destroying the actual cells themselves[70-80]. This is of particular importance because direct cellular biopsy may be difficult or otherwise unattainable for screening high-risk populations, such as screening for HCC in CHC patients. It is highly anticipated that future studies on exosomal ncRNAs in different stages of HCC in HCV patients, which reflect the stepwise carcinogenic process from preneoplastic lesions to HCC may unveil better and reliable markers to aid in HCC early diagnosis among CHC patients and tracking of disease progression, which may directly benefit patients affected with HCC. Furthermore, there is a possibility that ncRNAs in exosomes may be taken by hepatocytes as a part of the cell-to-cell communication to spread HCV and promote carcinogenic signal transduction among hepatocytes, and therefore studies on exosomal ncRNAs associated with HCV-induced HCC may suggest novel molecular targets to help prevent and treat HCV-induced HCC patients.

In conclusion, the recent discovery of ncRNAs ushered in exciting and novel area to explore. Meanwhile, challenges to investigators are obvious: With respect to ncRNAs in HCV-induced HCC, in-depth knowledge on functional roles for ncRNAs in HCV-induced HCC through well-designed studies are required to shed light on the molecular pathways of carcinogenesis and to aid in truly exploiting the potential of ncRNAs to serve as molecular targets or markers with a real value. Would it be possible in the future to use exosomal ncRNAs from body fluids such as blood, ascites or urine, for screening and early diagnosis of HCC among HCV patients at high-risk? Could we successfully slow or prevent the development of HCC in HCV patients using selected ncRNA antagomirs or mimics as novel approaches?

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Hepatitis C virus control among persons who inject drugs requires overcoming barriers to care

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Abstract

Despite a high prevalence of hepatitis C virus (HCV) infection, the vast majority of persons who inject drugs (PWID) have not engaged in HCV care due to a large number of obstacles. Education about the infection among both PWID and providers remains an important challenge as does discrimination faced by PWID in conventional health care settings. Many providers also remain hesitant to prescribe antiviral therapy due to concerns about adherence and relapse to drug use resulting in reinfection. Presently, however, as a result of improvements in treatment efficacy combined with professional society and government endorsement of HCV treatment for PWID, a pressing need exists to develop strategies to engage these individuals into HCV care. In this article, we propose several strategies that can be pursued in an attempt to engage PWID into HCV management. We advocate that multidisciplinary approaches that utilize health care practitioners from a wide range of specialties, as well as co-localization of medical services, are strategies likely to result in increased numbers of PWID entering into HCV management. Pursuit of HCV therapy after stabilization through drug treatment is an additional strategy likely to increase PWID engagement into HCV care. The full impact of direct acting antivirals for HCV will only be realized if innovative approaches are pursued to engage all HCV infected individuals into treatment.

Key words: Treatment of hepatitis C; Viral infection; Human immunodeficiency virus; Hepatitis C virus coinfection; Persons who inject drugs; Obstacles to treatment

Core tip: Despite persons who inject drugs (PWIDs) representing the majority of the hepatitis C virus (HCV) disease burden, few receive treatment for HCV. Barriers to treatment uptake exist at multiple levels. Co-localization of HCV management with substance abuse facilities may result in greater treatment uptake for PWID.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease affecting more than 120 million people worldwide[1,2] and at least 3.2 million in the United States[3-5]. Among HCV-exposed individuals, up to 80% will develop chronic infection that can ultimately lead to hepatic fibrosis, cirrhosis, hepatocellular carcinoma and death[6]. The prevalence of cirrhosis is estimated to increase from 25% in 2010 to 45% by 2030 in untreated patients with chronic HCV infection, and liver-related deaths are projected to increase by 175% over the next decade[7]. Currently, HCV is the leading indication for liver transplantation in the United States[8].

As the virus is most effectively transmitted via blood, injection drug use is currently the primary route of HCV transmission in the United States and other developed countries. Among persons who inject drugs (PWID), estimated HCV prevalence ranges from 30% to 70%, depending on frequency and duration of use, while incidence ranges from 16% to 42% per year[9,10]. Additionally, up to 20% of human immunodeficiency virus (HIV)-infected PWID in the United States are co-infected with HCV[11]. A recent study predicted that for a PWID population with 20% baseline chronic HCV prevalence, treatment rates of 5, 10, 20 or 40 per 1000 annually can lead to a 15%, 30%, 62% and 72% reduction in prevalence after 10 years, respectively[12]. The same authors have also estimated that novel treatments, expected to result in viral clearance rates of 90%, can halve HCV prevalence of 25%, 50%, and 65% within 15 years with treatment rates of 15, 40, or 76 per 1000 PWIDs annually[13]. Therefore, addressing HCV infection among PWID is a crucial step toward its successful control and prevention.

Despite the fact that PWID represent the majority of the HCV disease burden in developed countries, only 21%-65% have been evaluated for HCV, with less than 20% of evaluated patients receiving treatment[14-16]. Moreover, while the majority (> 70%) of PWID initially express willingness to undergo HCV treatment, only a minor percentage (1%-6%) actually receives therapy[14,16,17]. A variety of factors limit enrollment of PWID into HCV care and treatment. Identification of these barriers is therefore a key step toward formulating interventions to increase access to HCV care for PWID. Our goal in this article is to highlight the obstacles to providing HCV care to PWID and to propose interventions by which these barriers can be overcome.

BARRIERS TO HCV TREATMENT IN PWID

Obstacles to providing HCV care to PWID emanate from patients, health care providers and the health care system[18] (Table 1). One of the most important patient level obstacles to receiving care is lack of HCV-related knowledge resulting in a low perceived need for treatment. Between 65%-75% of HCV-infected patients are unaware of their status[19]. While many patients are aware that treatment for HCV exists, few are cognizant that it is curative. Some PWID are reluctant to undergo liver biopsy, an invasive procedure that has been frequently required prior initiation of HCV treatment. The presence of needles that are required for interferon injection might also be an obstacle to treatment in some persons who previously injected drugs. Additionally, many PWID perceive treatment-related side effects to be worse than the virus itself. Finally, mistrust of the health care system and difficulty keeping medical appointments may also contribute to PWID's unwillingness to initiate HCV therapy[14]. PWID are also more likely to be uninsured, have limited access to health care services, be affected by poverty, and have reduced social support[20].

Table 1 Most common barriers to engagement of persons who inject drugs into care for hepatitis C virus infection

Domain	Specific barrier
Patient-level	Low perceived treatment need
	Fear of side effects
	Lack of knowledge of serostatus
	Fear of liver biopsy
	Needles may promote relapse
	Coexisting mental health diagnosis
	Lack of insurance, poverty, low socioeconomic status
Physician-level	Concerns about reinfection
	Biases against PWID
	Adherence concerns
	Dual diagnoses
Health system-level	Navigation can be complex
	Mistrust between PWID and medical community
	High cost of HCV treatment
	Stigmatization in health care venues

HCV: Hepatitis C virus; PWID: Persons who inject drug.

Provider barriers also contribute to low rates of treatment provision to PWID. Patients who report injecting drugs are less likely to be referred for HCV evaluation and less likely to receive HCV treatment[21,22]. Many health care providers remain hesitant to treat patients with a history of drug use due to concerns about adherence to the therapeutic regimen. Some providers avoid treatment of PWID due to the misconception that reinfection occurs at a high level following relapse to injection drug use[23]. Finally, people with drug addiction have been perceived as challenging patients because they are more likely to be dually diagnosed with psychiatric co-morbidities, such as depression and anxiety, compared to non-addicted individuals[24].

The health care system itself may pose numerous obstacles to HCV treatment of PWID. The United States health care system is complex and the referral and scheduling process, as well as insurance and payment issues, can be difficult to navigate. Long-seated, distrusting relations between PWID and the medical community have contributed to feelings of stigmatization among those seeking HCV treatment. PWID often experience health care providers as judgmental, unresponsive to their medical needs, and disdainful, all of which serve as systemic barriers to care.

Finally, high cost of HCV therapy is another treatment barrier. For example, the estimated total cost of

telaprevir-based therapy, including the cost of side effect management, can be as high as $147000[25]. Although this problem is not specific to PWID, it certainly affects them to a greater extent compared to general population, particularly as PWID are more likely to be uninsured and to have less financial resources.

Excluding PWID from HCV treatment contradicts current recommendations issued by several United States governmental and relevant professional organizations. Governmental bodies, including the Institute of Medicine (IOM)[26] and the Department of Health and Human Services (HHS)[27], now advocate for increased awareness and resources to address the issue of disparities in HCV treatment for PWID. Professional organizations such as the American Association for the Study of Liver Disease (AASLD)[28], have stated in their guidelines that PWID should be treated for HCV. Yet despite these recommendations, PWID are frequently excluded from therapy by the health care system.

OVERCOMING THE OBSTACLES TO HCV TREATMENT FOR PWID

Through advances in HCV management, we are now experiencing partial resolution of the obstacles to HCV treatment among PWID. The rapid acceleration of HCV treatment toward an all oral regimen with improved efficacy and fewer adverse effects will likely result in the elimination of the liver biopsy as a requirement to initiate treatment. Additionally, the avoidance of needle exposure associated with interferon injection would eliminate anxiety among persons who no longer inject drugs. The onus now moves toward strategy development to address other obstacles in the management of HCV in PWID.

As patient-related obstacles can derive from misconceptions and lack of HCV-related knowledge, appropriately designed educational interventions could prove beneficial in promoting HCV care and treatment. Unfortunately, while nationwide surveys in the United States have documented that most opioid agonist treatment (OAT) facilities provide at least some form of HCV education[29,30], patients infrequently avail themselves of these opportunities[31]. Increased awareness of potential benefits of such programs and the addition of patient incentives, such as financial compensation or travel stipends, might increase participation. Peer support groups, directed by treatment-experienced patients, could encourage treatment acceptance and provide emotional support through shared treatment experiences. Support from mental health and allied health professionals to assist with procurement of social and mental health services, temporary disability, accessing Medicaid, and obtaining transportation, may potentially increase involvement in HCV treatment. These interventions can be incorporated into an individualized treatment plan to maximize adherence rates and successful outcome achievement.

Other obstacles to provision of HCV care and treatment result from lack of HCV-related knowledge and misconceptions among health professionals regarding PWID. These barriers may be overcome by provider education about PWID or by close collaboration between health care providers from diverse specialties[32]. Involvement of a multidisciplinary team consisting of representatives of hepatology, addiction medicine, generalists, and mental health experts in the treatment of HCV for PWID has been shown to result in increased treatment efficacy[32]. Besides direct interaction for the purposes of patient care, mentoring programs conducted between HCV specialists, substance abuse treatment staff, and peers could increase knowledge and build the skills necessary to treat this population. Mentoring programs could be conducted in person or via telemedicine.

A recent meta-analysis demonstrated that HCV treatment outcomes among PWID were improved among those treated for opioid addiction compared to untreated individuals[32]. In addition, rates of successful treatment outcomes for PWID were shown to be almost identical to outcomes achieved in registration trials[32,33]. However, while occasional drug use does not impact on adherence, treatment completion or treatment efficacy, frequent drug use (daily or every other day) does[34]. Consequently, successful outcomes for HCV are more likely to be achieved if PWID who inject frequently are initially stabilized for their addiction and subsequently undergo HCV therapy.

By co-localizing both HCV preventive and treatment services at venues where PWID receive care for drug addiction, uptake of HCV services might increase. For example, due to annual HCV serologic testing in some OAT facilities, HCV-infected patients have been more readily identifiable. At present, however, offsite referral to HCV specialty-care clinics is a common practice among drug treatment providers[29,35]. However, its effectiveness is limited as the majority of referred patients often fail to schedule or appear at appointments[14,36,37]. Yet, OAT facilities that do offer on-site HCV evaluation and treatment have achieved improved outcomes[38-41]. Similar findings have been previously reported for HIV-infected PWID, many of whom voluntarily use primary care services if they are offered onsite in OAT facilities[42]. Unfortunately, a recent study of substance abuse treatment programs affiliated with academic medical centers conducted through the National Drug Abuse Treatment Clinical Trials Network found a significant lack of comprehensive HCV counseling, testing, and treatment both on-site or by referral[43]. The same programs, however, offered significantly more HIV/AIDS-related health services[44].

OAT facilities that do offer integrated HCV care programs may also provide comprehensive on-site primary care services administered by health care providers with training in diverse disciplines including infectious diseases, hepatology, addiction medicine, and mental health[45-47]. Many of these programs also offer active case management and have diverse staff consisting of physicians, physician assistants, nurse practitioners, nurses, counselors, and social workers. To improve adherence, some programs utilize directly observed therapy as well

as offering counseling sessions, motivational interviewing, peer-based support groups, and HCV-related education[45,47-50]. Improvement over offsite referral has also been achieved through an integrated model combining addiction medicine physicians with hepatologists in a viral hepatitis clinic[51].

Finally, overcoming the financial obstacles for HCV treatment will not be easy, especially in developing countries. In the United States, health care reform will promote integration of specialty services into primary care, promote prevention, and will likely provide an opportunity for development of innovative models for previously medically-marginalized populations such as PWID. In contrast, in developing countries, pharmacy assistance programs will most likely be necessary in order to enable patients to access novel HCV treatments.

PARALLELS BETWEEN HIV AND HCV

The issue of increasing awareness and funding for HCV treatment among PWID has many similarities to HIV; indeed, HIV treatment is often touted as one of the great medical successes of our time. As the gravity of the emerging HIV epidemic became apparent in the 1980s, national attention and subsequent funds were directed toward combating the infection. Although similarities exist between both viral infections, so do important differences. For example, HCV is curable in a majority of cases while HIV presently requires costly lifelong treatment. Prevention activities among PWID that have been highly effective in controlling HIV have not been as effective in the control of HCV, largely due to limited funding and advocacy[52]. Additionally, the ultimate consequences of HCV infection, such as development of end-stage liver disease, hepatic decompensation, or hepatocellular carcinoma leading to liver transplant and subsequent lifelong immunosuppression, are largely preventable through screening and subsequent treatment. With implementation of improved therapies, the HCV field hopes to achieve the same levels of success accomplished by the HIV field.

CONCLUSION

As many HCV-infected PWID acquired the virus decades ago, they suffer from cirrhosis and other complications of end-stage liver disease with increasing prevalence. Therefore, strategies to increase HCV care and treatment among PWID are critically needed. Achieving higher treatment rates among this population will require overcoming existing barriers at the patient, provider, and institutional levels. Co-localization of HCV management with substance abuse treatment may be a strategy that could facilitate HCV diagnosis as well as promote treatment acceptance and adherence. This approach would reduce the prevalence of end-stage liver disease, viral transmission, and HCV-associated mortality. Additionally, early identification and treatment of HCV infection is more cost-effective compared to management of end-stage liver disease[53]. Tremendous advances are presently occurring in the HCV field, and we hope that PWID will be included in these changes.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Between Scylla and Charybdis: The role of the human immune system in the pathogenesis of hepatitis C

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Abstract

Hepatitis C virus (HCV) frequently elicits only mild immune responses so that it can often establish chronic infection. In this case HCV antigens persist and continue to stimulate the immune system. Antigen persistence then leads to profound changes in the infected host's immune responsiveness, and eventually contributes to the pathology of chronic hepatitis. This topic highlight summarizes changes associated with chronic hepatitis C concerning innate immunity (interferons, natural killer cells), adaptive immune responses (immunoglobulins, T cells, and mechanisms of immune regulation (regulatory T cells). Our overview clarifies that a strong anti-HCV immune response is frequently associated with acute severe tissue damage. In chronic hepatitis C, however, the effector arms of the immune system either become refractory to activation or take over regulatory functions. Taken together these changes in immunity may lead to persistent liver damage and cirrhosis. Consequently, effector arms of the immune system will not only be considered with respect to antiviral defence but also as pivotal mechanisms of inflammation, necrosis and progression to cirrhosis. Thus, avoiding Scylla - a strong, sustained antiviral immune response with inital tissue damage - takes the infected host to virus-triggered immunopathology, which ultimately leads to cirrhosis and liver cancer - the realm of Charybdis.

Key words: Natural killer cells; $CD4^+$ T helper cells; Regulatory T cells; Interferon; Hepatitis C; Hepatic stellate cells; Hepatocytes; Immunoglobulin; Retinoic acid inducible gene-Ⅰ; Toll like receptors

Core tip: This topic highlight on the immunopathogenesis of chronic hepatitis C addresses changes in innate immunity (interferons and natural killer cells), adaptive immunity and immunoregulation (regulatory T cells). Our review provides a succinct but comprehensive overview and presents the concept, that effective antiviral immunity is associated with pronounced acute liver damage, while during chronic infection the arms of immunity will acquire new functions, which will cause and maintain tissue damage. Thus, the immune response becomes part of the mechanisms that eventually lead to progressive inflammation, liver cirrhosis and death in chronic hepatitis C.

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INTRODUCTION

Scylla and Charybdis were two immortal and irresistible sea monsters in Greek mythology believed to live on either side of the Strait of Messina between Sicily and Italy. Scylla was a six-headed supernatural creature - probably

reflecting a shoal that devoured whatever came within her reach, and Charybdis was a whirlpool off the coast of Sicily. Avoiding Charybdis meant passing too close to Scylla and vice versa. According to Homer, the Greek hero Odysseus opted for Scylla when passing the strait, and had to sacrifice six of his companions rather than to risk the loss of his vessel in the whirlpool. Thus, being "between Scylla and Charybdis" means to be forced to make a choice between two equally unpleasant evils.

This allegory matches the challenge of the human immune system when defending against a viral infection, such as hepatitis C which has a high potential to establish chronic persistence. On one hand a strong and efficient immune response rapidly clears the virus; accepting the risk of severe tissue damage from immune-mediated destruction. On the other hand a less vigorous response allows for viral persistence and facilitates a low-level smoldering inflammation, which eventually results in progressive liver disease and ultimately death of the individual. In line with this analogy, acute self-limited hepatitis C is frequently associated with symptomatic disease and jaundice, while chronic hepatitis C often establishes in the absence of any characteristic symptoms[1,2]. Studies in various expression systems (cell culture or transgenic mice) indicate that hepatitis C virus (HCV) is not directly cytopathic, and viral replication may occur in the absence of any detectable inflammatory reaction[3,4]. On the other hand, chronic hepatitis C is associated with liver cell damage and intrahepatic inflammatory infiltrates. Of note, hepatocellular damage coincides with the onset of an immune response during acute infection but not with that of viral replication[5]. Thus, activation of the immune response is a pivotal factor for the pathogenic processes in hepatitis C leading to progressive tissue injury. Ultimately, hepatic inflammation and progressive fibrosis in chronic hepatitis C may result in cirrhosis and carry a high risk for hepatocellular carcinoma.

BASIC FACTS

HCV is a hepacivirus of the *Flaviviridae* family. Its genome consists of a single strand positive sense RNA. After cell entry the viral genome is translated into a single polyprotein which is co- and post-translationally cleaved into structural and non-structural proteins by host peptidases and two virus-encoded proteases. Replication involves generation of an antigenomic replication intermediate, and probably intermediate double-stranded RNA (ds-RNA) products, which can trigger intracellular pattern recognition receptors. The new viral genomes are packaged into viral particles by the viral non-structural proteins, which then are released from hepatocytes in association with host lipoproteins. Thus, HCV circulates in blood as a lipoprotein-coated virus[6]. During replication HCV is sensed by pattern recognition receptors (PRRs) in the host cell which detect pathogen-associated molecular patterns within viral products. This process then leads to coordinated activation of innate and adaptive immune responses. Both arms of the immune response work together in an integrated fashion to recognize and defend against HCV infection.

Innate responses to HCV comprise both cellular responses, such as recognition of non-self by various types of natural killer (NK) cells and humoral components, such as induction of a variety of cytokines, especially interferons. These various elements of innate immunity act in a highly integrated fashion as do innate and adaptive immune responses. Thus, development of adaptive B and T cell immunity is shaped by the initial innate responses, in particular interferons and other inflammatory and immunoregulatory cytokines that are induced by viral invasion[7]. However, despite these immune defences, hepatitis C becomes chronic in about 70%-80% of acute infections[8]. Failing immunity and continued viral persistence lead to sustained inflammatory host responses which then become the key mechanism for tissue injury in chronic hepatitis C.

INNATE IMMUNITY IN HEPATITIS C

Three types of PRRs are known to detect HCV: (1) the retinoic acid inducible gene-Ⅰ (RIG-Ⅰ)-like receptors, RIG-Ⅰ and melanoma differentiation antigen 5, which sense viral RNA in the cytosol; (2) toll-like receptors (TLRs), such as TLR3, which detects ds-RNA fragments in the endosomal compartment; and (3) the non-traditional pattern recognition receptor protein kinase R (PKR), which binds ds-RNA binding and upon activation promotes interaction with mitochondrial antiviral signaling protein (MAVS) to trigger innate immunity[9].

RIG-Ⅰ signaling is initiated by binding of the HCV PAMP RNA which consists of an exposed 5'triphosphate and the 3'poly-U/UC-rich untranslated region of the HCV RNA[10,11]. These regions are located at opposite ends of the viral genome but are brought together by intra-genomic interactions. In this configuration the viral RNA comes into close contact with RIG-Ⅰ and induces conformational changes of RIG-Ⅰ. RIG-Ⅰ activation leads to the formation of a multi-component complex with MAVS (also termed interferon beta promoter stimulator protein 1 or card adaptor inducing interferon beta, cardiff). Finally, the interferon signaling cascade results in the activation of multiple transcription factors, such as interferon-regulatory factor-3 (IRF-3) and nuclear factor kappa B and production of multiple pro-inflammatory cytokines[12].

HCV dsRNA intermediates, which occur late in HCV replication, have been identified as ligands for TLR3[13]. TLR3 signals are transmitted by the adaptor molecule TIR-domain-containing-adaptor-inducing-interferon-β (TRIF) and also lead to production of interferons and pro-inflammatory cytokines[14]. TLR3 mediated interferon and cytokine responses are considered a secondary innate immune defense after initial RIG-Ⅰ activation to establish an antiviral state and trigger T cell recruitment in HCV infection.

The ligand for PKR is the structured RNA at the internal ribosomal entry site (IRES) of HCV RNA[15,16]. Binding of HCV RNA induces phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2α). In addition, RNA binding also triggers a kinase-independent signal transduction cascade involving MAVS which finally activates interferon-β and interferon-stimulated genes (ISGs)[9,16].

Although HCV can be detected effectively by RIG-Ⅰ, TLR3 and PKR, it frequently establishes chronic persistence in up to 80% of patients, because it has evolved several mechanisms to counter-act innate immunity. The multi-functional HCV NS3/NS4A protease is a key component of the HCV evasion strategy from innate immunity. Studies in Huh-7 cells indicate that HCV initially activates the RIG-Ⅰ pathway which is shut down as infection progresses and NS3/NS4 abundance increases[17]. In addition to proteolytically processing the HCV polyprotein, NS3/NS4A can block RIG-Ⅰ signaling, because it cleaves MAVS from intracellular membranes[18-21]. This cleavage prevents signal transduction, abrogates interferon induction and facilitates progression to chronic infection. However, other hepatotropic viruses, such as hepatitis A virus also encode proteases that can cleave MAVS but in general do not become chronic[22]. Thus, MAVS cleavage alone is not sufficient for viral chronicity. Nevertheless, cleavage of MAVS has been demonstrated in the livers of patients with chronic hepatitis C, and patients with cleaved MAVS revealed reduced interferon pathway activation, although this inverse correlation was rather weak[23].

The NS3/NS4A protease can also cleave TRIF[24], the adaptor protein of the TLR3 pathway, and the relative abundance of this protein is reduced after HCV infection, probably as a result of degradation following its cleavage by NS3/NS4A[25]. Although details are insufficiently understood at present, blocking of the TLR3 pathway by HCV also seems to contribute to establishing chronic infection. TLR3-independent sensing of RNA which signals *via* TRIF has also been described, and is likewise blocked by NS3/NS4A targeting of TRIF[26]. Finally HCV proteins E2, NS3, NS4A and NS5 provide several strategies to interfere both with PKR signaling and PKR-regulated inhibition of translation[27-29]. However, these interactions are complex and the exact mechanisms how they support HCV persistence are still unclear.

Continued triggering of PRR pathways in chronic hepatitis C is likely to contribute to immunopathology, such as hepatic inflammation, fibrosis progression and HCV-associated malignancy. In this context it is interesting to note that HCV proteins core and NS3 also trigger TLR 1-2 and 2-6 dimers[30,31], and there is evidence from genetic epidemiology and functional *in vitro* studies that HCV-TLR interactions might contribute to hepatic fibrogenesis and cirrhosis[32,33], development of liver cancer[34], HCV-associated autoimmunity and B cell lymphoma[35].

INTERFERONS

HCV recognition by PRRs ultimately leads to induction of antiviral cytokines termed interferons (IFNs). Type Ⅰ IFNs (several interferons-α and interferon-β) bind to the ubiquitously expressed type Ⅰ interferon receptor, while type Ⅲ IFNs [IFN-λ1 alias interleukin (IL)-29, IFN-λ2 alias IL-28A, IFN-λ3 alias IL-28B] have their own receptor consisting of the IL10R2 chain (IL-10 receptor beta chain) and a unique IFN-λ receptor chain with a limited expression mainly on hepatocytes[36,37]. The type Ⅱ interferon IFN-γ has its own IFN-γ receptor. All IFN receptors transmit signals from the cell surface to the nucleus *via* the Jak-STAT pathway to activate interferon stimulated genes (ISGs). Specifically type Ⅰ and Ⅲ IFNs induce IFN-stimulated gene factor 3 consisting of phosphorylated STAT1 and 2 proteins and IRF9 which activate the IFN-stimulated response elements (ISRE) of multiple genes contributing to antiviral activity[38-40].

IFN signaling is regulated by suppressors, such as suppressor of cytokine signaling and ubiquitin specific peptidase 18 (USP18) which provide important negative feed-back loops[41-43]. USP18 is a protease cleaving ISG15 from its target proteins, also including STAT1[44]. ISG15 is conjugated to STAT1 by the sequential action of several enzymes[45]. This so-called ISG-ylation and its de-conjugation by USP18 modify signal transduction pathways and immune responsiveness[46,47]. However, recently it has been recognized that USP18 suppresses IFN-signaling independently from its de-conjugating activity by interfering with the interaction between Jak1 and the type Ⅰ IFN receptor[48]. USP18 is a major mediator of unresponsiveness to type 1 IFNs in liver cells[49]. However, it does not inhibit signal transduction of type Ⅱ and Ⅲ IFNs[50].

Activation of the endogenous IFN system in the liver exerts little anti-HCV activity, and it has been well established that patients with high activation of the endogenous IFN system respond poorly to IFNα based therapies[51-55]. It has been proposed that expression of HCV proteins inhibits binding of activated STATs to ISRE[56], and Jak-STAT signaling was found to be inhibited both in HCV transgenic mice and liver biopsies from patients with hepatitis C[57,58]. Beyond that, phosphorylation and activation of STAT3 is involved in the antiviral IFN activity[59], and STAT3 expression was found to be reduced in HCV-infected livers[60]. Indeed, HCV core protein can prevent STAT3 phosphorylation[57,60,61], and this has been associated with HCV resistance to IFN-α[62]. Next, HCV-induced PKR activation inhibits cap-dependent translation of antiviral host proteins at the ribosomes owing to phosphorylation of eIF2α while production of HCV proteins is not impaired, because translation occurs *via* an IRES-dependent mechanism[63]. Of note, most studies on endogenous ISG induction in hepatitis C were based on steady state mRNA level measurements rather than determination of protein concentrations[51-55]. Finally, HCV proteins might directly inhibit ISG antiviral

effector functions apart from their inhibition of ISG translation. This concept is supported by experimental evidence from knock-out mice which demonstrated that expression of the USP18 leads to a long-term refractory state towards IFNα stimulation[49]. Likewise, strong USP18 expression was found in many hepatocytes of patients with chronic hepatitis C and high endogenous IFN activity, when histological specimens were studied[64]. At present the cellular sources and involved types of IFNs that maintain long-term ISG expression in chronic hepatitis C are still a matter of debate. IFN-λs are strong candidates as triggers of ISG induction in patients with chronic hepatitis C and endogenous activation of the IFN system, because, unlike all other IFN types, IFN-λ mRNA is readily detected in liver biopsies[53], and their action is not inhibited by USP18[50].

In patients with chronic hepatitis C endogenous ISG induction varies considerably between individuals, and this variability, as well as differential responsiveness to exogenous IFN-α is attributed to a combination of viral and host factors. For instance, difficult-to-treat HCV genotypes 1 and 4 induce high levels of endogenous IFN expression in hepatocytes resulting in an IFN-insensitive state that attenuates treatment responses[65]. Of note, endogenous ISG induction in Kupffer cells, the resident liver macrophages, is also a strong predictor of treatment responsiveness[66]. However, the relationship between baseline ISG induction and treatment outcome is opposite to that observed in hepatocytes: Virtually all non-responders lack baseline induction of ISGs whereas strongly induced ISG expression is found in responders[67]. This finding suggests that ISG induction in Kupffer cells may have a protective role for the host concerning both spontaneous HCV elimination and treatment outcomes.

Apart from viral factors genome-wide association studies have identified single nucleotide polymorphisms (SNPs) upstream of the *IFNL3* gene on chromosome 19q13, which are associated with outcomes of HCV infection both under IFN-based therapy of chronic hepatitis C[68-70] and disease evolution during acute HCV infection[71,72]. Although some initial studies failed to find a relationship between the SNPs and IFNL3 mRNA expression[71,73], it has meanwhile become clear that SNPs in this region alter IFN-λ expression levels[53,68,70,74-76], and the unfavorable minor alleles result in less *IFNL3* expression. Thus, it is quite unlikely that these SNPs simply reflect linkage disequilibrium with some other gene. However, the molecular and cellular mechanisms that underlie this association between outcomes of HCV infection and the *IFNL3* gene locus are not yet understood. It has been proposed that the unfavorable *IFNL3* variants may lead to compromised innate immune functions in particular with respect to natural killer cell activity[77,78]. However, given the fact that NK cells do not express type Ⅲ IFN receptors this hypothesis needs refining[79]. In addition, a dinucleotide polymorphism upstream of the *IFNL3* gene has been described, which can create or disrupt an alternative open reading frame giving rise to a new gene, termed *IFNL4*[80,81]. Although it has been proposed that loss of *IFNL4* expression should be protective against HCV, it is as yet not clear if the putative *IFN-λ4* gene product plays any role for differential immune responses to HCV infection.

NATURAL KILLER CELLS

NK cells constitute a first line of defence against viral infections. They rapidly recognize and lyse virus-infected cells, inhibit viral replication but also exert immune-regulatory functions. NK cells constitute approximately 30% of resident lymphocytes in a normal liver, and may account for as many as 60% of lymphocytes in HCV infection[82].

Activation of natural killer cells results from the integration of multiple activating and inhibitory signals *via* specific receptors. The most important NK cell receptors (and their cognate ligands) comprise the killer immunoglobulin-like receptor (KIR) family (ligands: HLA-A, -B and -C), the CD94-NKG2A/C complex (ligand: HLA-E), NKG2D (ligands: MIC-A and MIC-B and others) and the natural cytotoxicity receptors NKp30, NKp44 and NKp46[83]. In addition, part of these receptors also exerts immune-regulatory functions in subsets of T lymphocytes. NK cells are activated, when there is a relative reduction of inhibitory signals, *e.g.*, down-regulated MHC class I expression on virus-infected cells, or a relative increase in signals from activating receptors, *e.g.*, binding of antibody-coated antigens[84]. However, conventional MHC class I expression is not substantially reduced in hepatitis C, and it has been proposed that NK cell functions might be altered by binding of HCV-derived peptides to non-polymorphic restriction molecules, such as HLA-E[85,86]. NK cells are recruited to inflammatory sites by a variety of chemokines and can also be stimulated by cytokines, such as IFN-α and ILs 8, 12, 15 and 18[87].

Activated NK cells with potent de-granulation and substantial cytokine production have been described in acute HCV infection[88,89], and there is accumulating evidence to suggest that NK cells play an important role in the antiviral immune response to hepatitis C and later on also in the immune-mediated pathogenesis of chronic hepatitis C. NK cells can inhibit HCV replication *in vitro* both by IFN-γ mediated non-cytolytic as well as granzyme/perforin and TRAIL-mediated cytotoxic mechanisms[90]. While HCV-infected hepatocytes up-regulate expression of TRAIL receptors[91], *in vivo* IFNγ-mediated clearance of HCV might be more important than direct cytolysis, because cytolytic elimination of all HCV-infected hepatocytes would lead to extensive liver damage[92]. Multi-functional NK cells are also detectable early after HCV exposure in health-care workers and iv drug users, who do not proceed to develop acute hepatitis; suggesting a potentially protective role of NK cells in early HCV infection[93,94]. Further support for a pro-

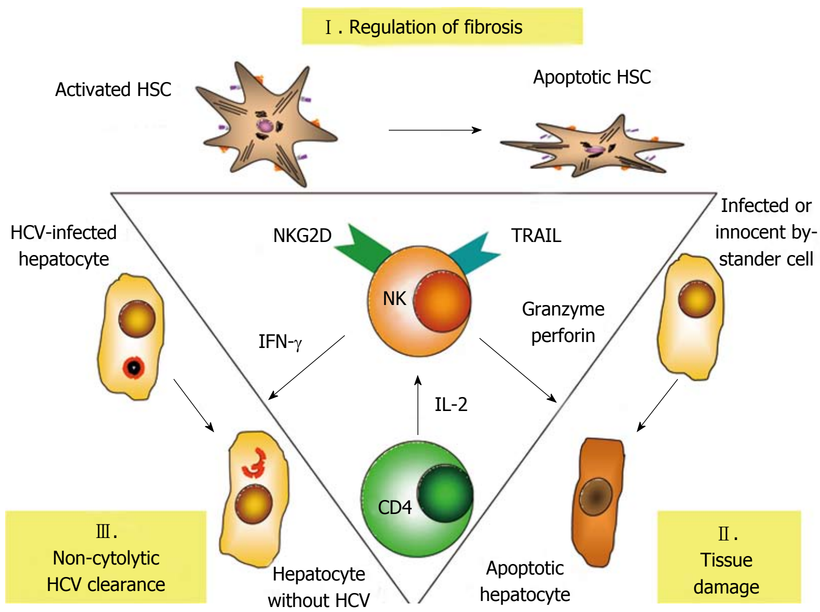

Figure 1 Central role of natural killer cells in the pathogenesis of hepatitis C. Natural killer (NK) cells regulate fibrosis by killing of activated hepatic stellate cells (HSC), which trigger NK cell activation *via* natural killer cell receptor with extracellular C-type lectin domains (NKG2D) signalling. The release of granzyme/perforin and cytotoxic cytokines, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce tissue damage. Interferon-γ (IFN-γ) released from NK cells can clear hepatitis C virus (HCV) infection from infected hepatocytes without cytolysis. On the other hand NK cell activity is critically dependent on sufficient supply with interleukin 2 (IL-2) from $CD4^+$ T cells.

tective role of NK cells in HCV infection comes from genetic studies, where genes encoding the inhibitory receptor KIR2DL3 and its ligand human leucocyte antigen group 1 (HLA-C1) seem to favour both spontaneous and treatment-induced elimination of HCV[95,96]. Since the affinity between inhibitory KIR2DL3 and HLA-C1 is weaker than all other combinations, it is reasonable to assume that a lower threshold of activation is needed to trigger KIR2DL3 NK cell responses in HLA-C1 homozygous individuals[88,97]. Finally, NK cells can become more activated upon IFN-based therapy and may contribute to HCV elimination by TRAIL-mediated cytotoxic mechanisms[98]. Interestingly, responsiveness in this setting again depends on the endogenous IFN-α activation state, since a rapid first phase HCV decline is associated with strong induction of STAT1 phosphorylation, whereas non-responders exhibit reduced STAT1 induction[99]. Chronic exposure of NK cells to IFN-α results in preferential STAT1 over STAT4 phosphorylation, which is associated with increased STAT1-dependent cytotoxicity but reduced STAT4-dependent IFN-γ production[99-101]. These findings correspond to NK cell phenotypes and functional differentiation seen at later stages in IFN-α responders and non-responders[100,102], although patients who achieve a sustained virological response also exhibit substantial NK cell cytotoxicity[103].

NK cells in chronic hepatitis C have been reported to also express altered patterns of NK receptors (Figure 1). Although reported patterns are somewhat inconsistent and may vary between peripheral blood and the liver, altered expression on NK cells has been reported for receptors NKp30, NKp44, NKp46, NKG2A, NKG2C, NKG2D and CD122[100,104-107]. In addition, NK cells express the tetraspanin CD81, a co-receptor of HCV, and *in vitro* binding of the HCV envelope 2 (E2) protein to CD81 has been shown to block antiviral functions of NK cells and to alter their migratory behaviour[108-111]. However, the experimental setting of these studies involved cross-linking of HCV E2 on plastic plates, whereas NK cells exposed to intact virions did not exhibit altered functionality[112]. Thus, it remains to be elucidated if cross-linking of CD81 by HCV E2 affects functions of NK cells to facilitate chronic infection. A particularly interesting NK cell receptor is NKp46, since it is considered a major activating receptor in hepatitis C, which also has a role in the regulation of adaptive immunity: High expression of NKp46 defines a NK cell subset with high cytotoxic activity and IFN-γ production that accumulates in the liver in chronic hepatitis C[113,114]. Of note, recently Pembroke *et al*[115] confirmed intrahepatic enrichment of NKp46+ NK cells in chronic hepatitis C and reported a high (> 80%) frequency of NKp46+ cells in the liver to be associated with pronounced inflammation in histology. Another important finding of this study was the observation, that expression of NKp46 could predict responses to IFN therapy. Patients with chronic hepatitis C, who successfully cleared their HCV infection, had lower mean frequencies of activated NKp46+ NK cells than patients who did not respond to therapy. The possible identification of NKp46 as a marker of both IFN-un-responsiveness and hepatic inflammation bears some similarity to the paradoxical relationship between IFN-un-responsiveness and high baseline ISG expression and may be linked to chronic endogenous interferon exposure. On the other hand, unlike Pembroke *et al*[115] the group of Golden-Mason[113] reported increased NKp46

expression in white female Americans as opposed to male African-Americans and proposed that a high proportion of functionally active NKp46+ NK cells could explain their higher response to IFN therapy. Thus, the precise role of NKp46+ NK still remains elusive.

Finally, NK cell-mediated cytotoxicity against hepatic stellate cells (HSC) may contribute to the regulation of intrahepatic fibrosis in hepatitis C. HSC store vitamin A, reside in the space of Dissé, and produce extracellular matrix proteins upon activation, *e.g.*, upon TLR stimulation, exposure to cytokines or reactive oxygen species[116]. HSC activation leads to trans-differentiation into myofibroblasts, which in the mouse also alters the balance in the expression between activating and inhibitory NK cell receptor ligands, so that they become target cells for NKG2D-, TRAIL- and granzyme-mediated killing by NK cells[117,118]. NKG2D- and TRAIL-mediated killing by NK cells has now also been reported for human HSC in chronic hepatitis C[119], and CXCR3 + CD56^{Bright} as well as NKp46+ NK cells express particularly high cytotoxic capacity against HSC in chronic hepatitis C[114,120]. Importantly, when other processes, such as CD4 T cell depletion in HIV/HCV co-infection interfere with the regulation of hepatic fibrosis by NK cells, this may result in accelerated fibrosis progression[121].

ADAPTIVE IMMUNITY IN HEPATITIS C

A coordinated immune response involving both antibodies and T cell responses is normally required for efficient adaptive immunity. However, in hepatitis C the role of antibodies is complex: Circulating antibodies against structural and non-structural components are generated in virtually all patients irrespective from the outcome of HCV infection. A rapid induction of neutralizing antibodies early in the course of hepatitis C has been demonstrated to contribute to HCV clearance[122], but broad antibody responses usually occur at the stage of chronic infection and are not neutralizing[123,124]. Neutralizing antibodies frequently recognize the HCV envelope proteins[125-127]. However, these proteins have a high degree of mutational diversity, so that antibody responses are frequently directed against only a single strain or are easily evaded by viral mutations[124]. It is also quite likely that glycosylation of HCV proteins and the close association of the virus with lipoproteins further prevent antibody recognition. HCV antibodies are not required to clear HCV infection as has been demonstrated in patients with hypo-gammaglobulinemia[128]. HCV antibodies gradually disappear after successful HCV elimination[129]. Conversely, there is circumstantial evidence that HCV-specific cellular immune responses can protect individuals at high risk for hepatitis C without seroconversion[123,130,131]. Thus, adaptive cell-mediated immunity is considered a key mechanism for resolution of primary HCV infection[132]. Cell-mediated immunity involves CD8$^+$ cytolytic T lymphocytes (CTL), which recognize linear HCV peptides of 8 to 11 amino acids in length bound to self HLA class I molecules, and CD4$^+$ T helper lymphocytes, which respond to longer viral peptides bound to class Ⅱ molecules. Single source outbreaks further support a clear relationship between distinct HLA types and the outcome of HCV infection: patients with HLA-A3, HLA-B27, and HLA-B57 exhibit greater chances to develop protective immunity, thus strengthening the importance of effective antigen presentation and the generation of efficient antigen-specific T cell responses for immune control of HCV infection[133-136].

T CELL RESPONSES

The most conclusive experiments to suggest an important role for T cells in protective immunity against HCV stem from chimpanzee experiments: Depletion of CD8$^+$ T cells in animals, which had recovered from previous hepatitis C, resulted in prolonged viraemia, and viral clearance was correlated to recovery of HCV-specific CD8$^+$ T cells[137]. Likewise, depletion of CD4$^+$ T cells resulted in abrogation of a previously protective immune response[138]. In acute hepatitis C strong HCV-specific CTL[139,140] and TH1 type CD4$^+$ T helper cell responses[141] have consistently been reported to be closely associated with a self-limited course of HCV infection. Moreover, several groups have reported an inverse relationship between the strength of the CTL response and HCV viral loads[142-144] further suggesting that in principle it is possible for cellular immunity to control HCV infection[145]. A substantial proportion of individuals who ultimately develop chronic hepatitis C also generate HCV-specific CD4(+) and CD8(+) T cell responses during the early acute phase of infection and may transiently gain some control over HCV[140,146-149]. However, early T cell responses decline to almost undetectable levels later on, and initial control over HCV replication is lost. If present, HCV-specific CD4$^+$ and CD8$^+$ T cells are detected at only low frequency in peripheral blood although they are somewhat enriched in the liver[150,151]. Thus, chronic hepatitis C is characterized by a progressive functional exhaustion and ultimately loss of HCV-specific CD4$^+$ and CD8$^+$ T cells[152,153].

Exhausted T cells exhibit a couple of characteristic abnormalities: They show increased expression of inhibitory receptors, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing molecule 3, corresponding to up-regulated expression of their cognate ligands in the liver[154-159]. Conversely, functional recovery of HCV-specific T cells can be achieved experimentally by the combined blockade of CTLA-4 and PD-1 signalling[157,160].

HCV replicates by an RNA-dependent RNA polymerase which has a high error rate and consequently generates considerable genomic diversity of HCV and T cell escape mutations. Mutations that affect CD8$^+$ T cell epitopes and proteasomal processing have been observed in several HCV single source outbreaks[161-163].

Due to the exhausted state of T cells new epitope variants rarely elicit strong $CD8^+$ T cell responses at this stage of infection, and consequently further escape mutation to secondary epitopes are selected infrequently in man and the chimpanzee[146,164,165]. Protective T cells seem to target epitopes that do not allow for escape mutations owing to the associated loss of viral replication fitness[133-135]. Conversely, T cells that are not stimulated any more after HCV viral escape, do not show features of exhaustion[166]. Thus, prolonged exposure appears to be the mechanism that leads to T cell dysfunction in chronic hepatitis C, and T cell exhaustion in hepatitis C seems to follow the same pattern as has been first described in mice for lymphocytic choriomeningitis virus (LCMV) infection[167,168]. In this model, persistent high level viremia can be established in susceptible mouse strains by pathogenic virus variants. Initially, mice develop a robust T cell response but fail to eliminate the virus and subsequently exhibit a gradual decline of $CD8^+$ and $CD4^+$ T cell responses. T cells undergo T cell exhaustion in this model, and first lose production of IL-2, a cytokine which supports T cell proliferation. Then, cytotoxicity and production of tumour necrosis factor alpha and IFN-γ are lost sequentially. Finally, intracellular expression of pro-apoptotic factors, such as Bcl2-interacting mediator (Bim), is up-regulated both in the LCMV model and hepatitis C[169]. In analogy to the LCMV model, virus-specific $CD4^+$ and $CD8^+$ T cell responses decline in chronic hepatitis C but full exhaustion with deletion of antigen-specific CD8 T cells does not occur, because at least *in vitro* T cell responses can be rescued.

REGULATORY T CELLS

Recently $CD8^+$ T cells have been reported in the livers of patients with chronic hepatitis C which were considered to represent $CD8^+$ regulatory T cells, because they secrete IL-10 and suppress *in vitro* proliferation of liver-derived T cells[170]. In general, regulatory T cells (Tregs) actively control induction and activity of other immune cells by suppressing their functional activity *via* contact-dependent mechanisms and by release of immunosuppressive cytokines, such as IL-10 and transforming growth factor beta. The major cell type with these properties constitutes $CD4^+CD25^{high}CD127^-$ T cells, which express the transcription factor Foxp3 (forkhead box P3). They can be divided into thymus-derived natural regulatory T cells, that prevent autoreactivity to self-antigens and induced regulatory T cells, that are generated in the peripheral immune system as a regulatory response to antigenic stimulation. $Foxp3^+$ Tregs were rarely detected in acute hepatitis C[171] and they are also not found in patients who had managed to resolve HCV infection[172], suggesting that effector T cells in acute and self-limited hepatitis C are not under active suppression by Tregs. In chronic hepatitis C, however, numbers of $CD4^+$ Tregs were increased in the peripheral blood of patients, and depletion of $CD4^+$ $CD25^+$ T cells was associated with increased numbers and function of $CD8^+$ T cells in *in vitro* assays[173-176]. Such regulatory T cells may reduce inflammatory activity and are considered to contribute importantly to preventing immune-mediated pathology in chronic hepatitis C. Functional analysis of regulatory T cell clones generated from patients with chronic hepatitis C revealed that Tregs were directed against HCV antigens and showed the same pattern of HLA class Ⅱ restriction and epitope specificity as effector T cells[172]. Importantly, Treg clones from chronic hepatitis C inhibited *in vitro* proliferation and IFN-γ production of autologous reporter T cells *via* release of inhibitory cytokines, such as IL-10 and IL-35. Of note, intrahepatic regulatory T cells in chronic hepatitis C also produced substantial amounts of IL-8, and isolated Tregs as well as Treg clones activated fibrogenic genes of hepatic stellate cells *in vitro*[177]. High intrahepatic IL-8 mRNA levels in chronic hepatitis C have been linked with progression of fibrosis[178,179] and $CD4^+$ Tregs are enriched in the liver[175,177,180-182]. Moreover, some but not all studies also reported a correlation between numbers of intrahepatic Tregs and the stage of fibrosis. Beyond that, IL-8 counter-acted the antiviral activity of IFN-α in the replicon model by down-regulation the expression of ISGs[183,184]. Moreover, *in vitro* studies suggest that part of the superior antiviral activity in IFN/ribavirin combination therapy may be due to preferential inhibition of Tregs by ribavirin[185] (Figure 2).

Thus, once the immune system has failed to clear HCV infection, regulatory T cells in chronic hepatitis C seem to exert multiple different effects: they dampen inflammatory responses associated with reduced antiviral activity of the immune system, facilitate HCV persistence, and also contribute to the regulation of fibrosis in the liver.

CONCLUSION

When an individual becomes infected with HCV, the immune system has to make a choice between Scylla and Charybdis. If it takes a course close to Scylla, it generates strong antiviral immune responses, which eliminates virus infected liver cells by the combined action of its several innate and adaptive defense mechanisms. This may cause extended liver damage and eventually liver failure. To avoid this risk, immune responses may be softer. Then, the virus has a chance to escape from control by immunity, and functions of innate and adaptive immune mechanisms become diverted owing to continued antigenic stimulation. An inflammatory state is induced, which, however, is refractory to stimulation by antiviral cytokines, and NK cells as well as cells in the adaptive immune system take over regulatory functions. Necro-inflammatory and pro-fibrotic activities maintained by diverted immune responses inevitably take a course towards Charybdis, and may ultimately result in liver cirrhosis, liver cancer and death of the individual. Thus, the immune system holds the steer to find the way between

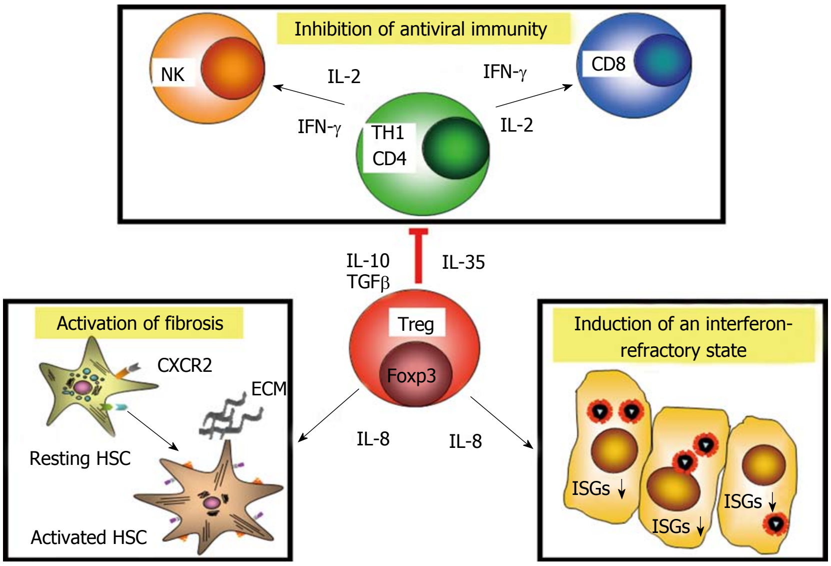

Figure 2 Multiple activities of hepatitis C virus-specific regulatory T cells in chronic hepatitis C. Regulatory T cell (Tregs) inhibit antiviral immunity *via* release of immunosuppressive factors, such as interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and interleukin 35 (IL-35). Tregs in hepatitis C are also differentiated towards interleukin 8 (IL-8) production. Release of IL-8 binds to its receptors, such as CXCR2 on hepatic stellate cells (HSC), which become activated and produce extracellular matrix (ECM) components. IL-8 down-regulates interferon-stimulated genes in infected cells and induces an interferon (IFN)-refractory state, which also counter-acts antiviral immunity. ISGs: Interferon-stimulated genes.

Scylla and Charybdis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Tumor necrosis factor-α inhibitors and chronic hepatitis C: A comprehensive literature review

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Abstract

Tumor necrosis factor-α (TNF-α) inhibitors are known to increase reactivation of concurrent chronic hepatitis B, but their impact on the hepatitis C virus (HCV) is controversial. Some conditions of immunosuppression, such as liver transplantation, typically cause an increase in the rate of HCV evolution. Inhibition of TNF-α, a cytokine involved in the apoptotic signaling pathway of hepatocytes infected by HCV, could potentially increase viral replication. Currently available clinical data appear to contradict this hypothesis. A review of medical literature revealed that a total of 216 patients with HCV were exposed to one or more treatments with TNF-α inhibitors, with a median observation time of 1.2 years and 260 cumulative patient-years of exposure. Only three cases of drug withdrawal due to suspected HCV liver disease recrudescence were reported. Treatment with TNF-α inhibitors in patients with HCV infection appears to be safe in the short term, but there are insufficient data to assess their long-term safety. Universal screening for HCV before beginning treatment with TNF-α inhibitors is currently controversial. The presence of HCV is not a contraindication to therapy with TNF-α inhibitors, with the exception of cirrhotic patients. In cases of cirrhosis, the benefit/risk ratio should be evaluated at the individual level. Prior to treatment with TNF-α inhibitors, patients with HCV should be referred to a hepatologist to determine the necessity of hepatic disease assessment, using liver biopsy or non-invasive methods, and the potential indication for antiviral therapy. In patients with HCV infection who are treated with TNF-α inhibitors, liver function monitoring every three months is advised.

Key words: Infliximab; Etanercept; Adalimumab; Hepatitis C virus; Rheumatoid arthritis; Inflammatory bowel disease; Psoriasis

Core tip: Our review summarizes data on patients with hepatitis C exposed to tumor necrosis factor-α (TNF-α) inhibitors, thus building a stronger safety profile than previously reported. A comprehensive paragraph on the pathway of TNF-α in hepatitis C virus (HCV) and an overview on immune-mediated damage induced by TNF-α inhibitors (cryoglobulins, autoimmune hepatitis) have been also included. Some controversies regarding the universal screening and monitoring of HCV-RNA were also addressed.

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INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a cytokine involved

in the pathogenesis of inflammatory diseases and in the immune-mediated response to infections, especially against intracellular pathogens. Drugs targeting and inhibiting the biological activity of TNF-α, such as infliximab, etanercept and adalimumab, are increasingly used for the treatment of immune-mediated diseases such as rheumatoid arthritis, inflammatory bowel diseases and psoriasis[1]. TNF-α inhibitors increase susceptibility to new or reactivation of concurrent infections. Thus, before its use for therapy, a screening for tuberculosis (with chest radiography and an interferon-gamma release assay) and certain viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus, and herpes virus is recommended[2].

The potential risk of reactivation of HBV infection during TNF-α inhibitor therapy is well established. Animal studies have demonstrated that TNF-α plays a key role in clearing HBV from infected hepatocytes by synergizing with interferons (IFNs) in the suppression of viral replication[3,4]. TNF-α inhibitors can increase HBV replication and reactivate chronic hepatitis, both during and after discontinuation of treatment. It is worth noting that many patients receiving TNF-α inhibitors have been previously or simultaneously treated, even for long periods, with other immunosuppressant agents that further increase the risk of HBV reactivation[5]. Hepatitis reactivation has been reported in twenty-three hepatitis B surface antigen (HBsAg)-positive patients treated with TNF-α inhibitors in the absence of prophylaxis (inactive carriers or with unrecognized HBsAg seropositivity), including 9 cases of fulminant hepatitis, 4 deaths and 1 liver transplantation. Furthermore, three HBsAg-negative, hepatitis B core antibody (Anti-HBc)-positive patients presented HBsAg seroreversion followed by a hepatitis flare-up after administration of TNF-α inhibitors[6]. The protocol that is currently recommended, borrowed from other clinical situations of pharmacologically induced immunosuppression, includes prophylaxis with lamivudine of all inactive carriers during and for 6-12 mo following therapy with TNF-α inhibitors and quarterly monitoring of HBsAg in HBsAg-negative anti-HBc positive patients[7,8].

In the context of HCV infection, the potential risk of reactivation of infection during therapy with TNF-α inhibitors is controversial. Several clinical reports have shown that chronic hepatitis C usually evolves rapidly in some conditions associated with immunosuppression, such as co-infection with human immunodeficiency virus, hypogammaglobulinemia, and after bone marrow transplantation and, above all, liver transplantation[9]. In various other circumstances, *e.g.,* following chemotherapy, hepatitis flare-up does not occur during immunosuppression or after its suspension[10]. The inhibition of TNF-α, a cytokine involved in the apoptotic signaling pathway of hepatocytes infected by HCV, could potentially increase viral replication and worsen the course of chronic hepatitis[11]. In this review, we present an overview of the relationship between the TNF-α pathway and HCV, summarize the available evidence regarding the safety of TNF-α inhibitor usage in patients with HCV and provide suggestions for the management of therapy in this clinical setting.

TNF-α PATHWAY IN CHRONIC HCV INFECTION

The role of TNF-α in chronic HCV infection is not well understood. Serum levels of TNF-α and its soluble receptors (sTNF-R55 and sTNF-R75) are significantly higher in HCV-infected patients than in healthy subjects[12]. Serum levels of TNF-α correlate with serum transaminase levels, histological activity and fibrosis, but not with serum HCV RNA levels or viral genotype[13,14]. Laboratory studies have indicated that the HCV core protein has the potential to inhibit the TNF-α-mediated apoptotic signaling pathway, providing a selective advantage for HCV replication and avoidance of the host antiviral defense mechanism[15]. Thus, further suppression of TNF-α by biological drugs may pose a potential threat of excessive viral replication and worsening of chronic HCV infection. In contrast, some studies have postulated that the baseline overexpression of TNF-α is associated with reduced cell capability to respond to IFN signaling and, consequently, to reduced viral clearance[16]. Zein *et al*[17] conducted a controlled, double-blind, randomized, placebo trial assessing the effects of etanercept as adjuvant therapy to IFN alfa-2b for 24 wk plus ribavirin in patients with chronic hepatitis C. The 19 patients treated with etanercept achieved sustained virologic response at a significantly higher rate compared to the 25 controls, and treatment was associated with decreased incidence of the most common side effects associated with IFN and ribavirin. This phase Ⅱ study supported the assumption that etanercept may restore TNF-induced $CD4^+$ cell impairment and enhance antiviral effects of IFN and ribavirin combination therapy. Large studies of the effects of adjuvant etanercept on therapy with pegylated IFN and ribavirin are currently lacking.

Infliximab is a recombinant human-murine chimeric immunoglobulin-G1 (IgG1) antibody that specifically binds both soluble and membrane-bound precursor forms of TNF-α. Etanercept is a dimeric fusion protein that consists of the extracellular ligand-binding portion of the human 75 kDa TNF receptor linked to the Fc portion of the human IgG1, and binds only soluble TNF-α. Adalimumab is a human-derived recombinant IgG1 monoclonal antibody that binds to TNF-α and blocks the interaction between soluble TNF-α and cell-surface TNF receptors[18]. The limited data that are currently available are not sufficient for the assessment of the potential specific differences between the drugs regarding the effect on viral replication.

CLINICAL EVIDENCE OF THE SAFETY OF TNF-α INHIBITORS IN PATIENTS WITH HCV

We performed a comprehensive review of reports pub-

Table 1 Safety of tumor necrosis factor-α inhibitors in patients with hepatitis C virus

Drug	Patients with HCV infection (*n*)	Mean follow-up (yr)	Patients/yr exposure	Elevation in AST/ALT serum level > 3 ULN	Elevation in HCV-RNA (> 1 log above baseline)	Drug withdrawal due to liver toxicity
Etanercept	153	1.14	174.49	3[1]	5	2
Infliximab	40	1.59	63.64	2	4	1
Adalimumab	23	0.97	22.43	0	0	0

[1]Elevation of transaminases without concomitant increase of HCV viremia in two cases. HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ULN: Upper limit of normal.

lished in English between January 2000 and August 2013; patients were evaluated for the following variables: disease, comorbidities, TNF-α inhibitors, previous HCV treatment, concomitant immunosuppressive drugs, liver function tests, HCV-ribonucleic acid (HCV-RNA), histopathological liver findings (when available), complications and outcomes. Patients with HCV are excluded from participation in controlled clinical trials with TNF-α inhibitors. Next, available data regarding the safety of TNF-α inhibitors in patients with hepatitis C, as derived from several case reports and small retrospective cohort studies in the field of rheumatology, dermatology and gastroenterology, in addition to the already mentioned trial of Zein *et al*[17], were evaluated. These findings come from various clinical contexts, in terms of differing uses of concomitant immunosuppressive drugs (in most cases dermatologists tend to employ TNF-α inhibitors in monotherapy, while gastroenterologists and rheumatologists prescribe them in combination with other immunosuppressants), pre-treatment selection of HCV patients, monitoring protocols and differences in the threshold used for discontinuing treatment with TNF-α inhibitors. Furthermore, most of the evidence concerns the measurement of transaminases and viral load, with few reports including a histological evaluation before and after treatment.

Total of 216 patients with hepatitis C were treated with one or more TNF-α inhibitors, with a median observation time of 1.2 years and 260 cumulative patient-years of treatment, a measure of exposure that includes all patients treated and normalizes the different durations of treatment to one year (Table 1)[19-58]. The majority of the available safety data concern etanercept. Clinical evidence suggests that the role of TNF-α in the control of HCV replication is modest. Currently, only three cases of drug withdrawal due to clinical suspicion of a worsening of HCV liver disease have been reported. The viral load in most cases remains stable or decreases, and it is difficult to confidently attribute the few cases of serum HCV-RNA increase > 1 log above the baseline value to treatment with TNF-α inhibitors, considering the well-known virological profile of HCV, which shows spontaneous fluctuations > 1 log of HCV-RNA level in 5%-10% of patients[59]. Overall, TNF-α inhibitors do not increase transaminase levels or viral load in the short term in patients with hepatitis C. Furthermore, the administration of these drugs has allowed the concomitant use of IFN in some patients with hepatitis C in whom IFN had been previously discontinued due of a worsening of concurrent immunomediated diseases such as psoriasis, rheumatoid arthritis or other arthritis. In regard to the long-term safety of TNF-α inhibitors and their impact on the progression of liver fibrosis, the limited available data do not allow for the assessment of this issue. Another area of uncertainty is related to their use in cirrhotic patients; only two cases of patients with cirrhosis who received TNF-α inhibitors have been reported, by Zein and Abdelmalek[17,36], and both cases were without significant side effects.

Another potential concern is the possibility of immune-mediated liver damage induced by TNF-α inhibitors. Emergence of serum auto-antibodies is a common observation in patients treated with TNF-α inhibitors and presents an additional concern in patients with hepatitis C. In the absence of HCV infection, the auto-antibodies induced by such treatments are usually non-organ specific [anti-double-stranded-DNA (dsDNA), rheumatoid factors, anti-cardiolipin] and belong to the IgM class[60,61]. Vauloup *et al*[62] prospectively evaluated the induction of circulating auto-antibodies during therapy with TNF-α inhibitors in patients with HCV and observed induction of anti-nuclear and anti-dsDNA antibodies, but no induction of anti-tissue antibodies (anti-smooth muscle and anti-liver/kidney/microsome type 1), even in patients with actively replicating chronic hepatitis C. Induction of cryoglobulinemia was also a possibility, and HCV-related mixed cryoglobulinemia usually includes an IgM component. Auto-antibodies emerging during treatment with TNF-α inhibitors are usually clinically silent, possibly due to the low avidity of antibodies to their antigen. Seventeen cases of TNF-α-induced hepatitis without known past history of liver disease have been reported in the literature[63-78]. The majority of these cases are secondary to infliximab and resemble autoimmune hepatitis type 1 due to an increased prevalence among females, the more common elevation of autoantibodies related to autoimmune hepatitis type 1 (anti-nuclear, anti-smooth-muscle or anti-dsDNA), the presence of interface hepatitis at liver biopsy, and the strong response to steroid therapy. Some of these patients were subsequently able to tolerate etanercept, suggesting a different potential of the two drugs in inducing immune-mediated liver damage. Indeed, among patients with HCV infection, only one case of granulomatous hepatitis not associated to a rise of serum HCV-RNA, diagnosed after 7 mo of therapy with etanercept, has been reported[79]. A TNF-α blockade induces a cytokine imbalance that is rarely responsible

for inducing pulmonary, cutaneous, eye and even hepatic sarcoidosis. Overall, the incidence of autoimmune hepatitis induced by TNF-α inhibitors appears to be low and does not represent a contraindication in the treatment of patients with chronic hepatitis C.

Although observations of transaminase elevation have been documented in the package inserts of TNF-α inhibitors, no direct link between these drugs and liver toxicity has been established to date, with the exception of one single case of acute hepatitis during infliximab treatment, in which the liver biopsy showed signs of toxic damage (intralobular necrosis, ceroid-containing Kupffer cells)[80]. For this reason, TNF-α inhibitors present an attractive alternative therapy in some patients with autoimmune diseases, such as psoriasis or rheumatoid arthritis, which are routinely treated with other drugs with well established, likely more severe liver toxicity profiles (cyclosporine, acitretin, methotrexate, leflunomide).

CLINICAL MANAGEMENT OF TNF-α INHIBITORS IN PATIENTS WITH HCV

Many guidelines recommend screening by means of serum anti-HCV antibodies in all patients undergoing therapy with TNF-α inhibitors, emphasizing that a definitive decision on the safety of TNF-α inhibitors in cases of chronic HCV infection has not been made[81-83]. A study conducted in Ireland, a country with a low prevalence of HCV (< 1%), including 215 patients with psoriasis treated with TNF-α inhibitors documented a single case of positivity for antibodies to HCV with undetectable serum HCV-RNA. The authors concluded that, in areas with low prevalence of HCV infection, universal screening should be replaced by targeted screening based on the individual risk factors of each patient[84]. Other guidelines state that universal screening should not be definitively recommended, as the risk of HCV reactivation under immunosuppressive drugs appears to be very low[85,86]. Before beginning treatment with TNF-α inhibitors, assays for serum alanine aminotransferase (ALT), gamma-glutamyl-transferase and total bilirubin are recommended, bearing in mind that approximately 30% of patients with chronic HCV infection show persistently normal ALT levels[87]. In cases of anti-HCV positivity, assessment of HCV-RNA, HCV genotype, cryoglobulins, complete blood count, total protein, albumin, total cholesterol, prothrombin time, creatinine, and urine exam, as well as a liver ultrasound, are also recommended.

TNF-α inhibitors in patients with HCV are not contraindicated, provided that monitoring of liver function tests is performed every three months during treatment. Currently, there is uncertainty in the standards for viral load monitoring (quarterly or only in case of serum transaminase increase). Due to the absence of data regarding cirrhotic patients, TNF-α inhibitors should be used with caution in compensated patients, while they are contraindicated in patients with decompensated liver disease, considering the extremely high risk of potentially fatal severe infections. In cases of reactivation of hepatitis, patients should be referred to a hepatologist for a differential diagnosis and to consider the potential for TNF-α inhibitor treatment withdrawal.

CONCLUSION

TNF-α inhibitor treatment in patients with HCV appears to be safe in the short term, but there are insufficient data to assess their long-term safety. A potential concern related to the administration of these drugs is the induction of immune-mediated reactions that potentially involve the liver (cryoglobulinemic syndrome or autoimmune hepatitis), but the incidence of such reactions appears to be low. Universal screening for HCV before beginning treatment with TNF-α inhibitors is currently controversial. The presence of HCV is not a contraindication to therapy with TNF-α inhibitors, except in cirrhotic patients, in whom the benefit/risk ratio should be evaluated at the individual level before treatment is initiated. Before administration of TNF-α inhibitors, patients with HCV should be referred to a hepatologist for the evaluation of the liver disease stage through liver biopsy or non-invasive methods and the potential for antiviral therapy. Liver function tests are advised for patients with HCV at a frequency of every three months during treatment with TNF-α inhibitors.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Relationships between lymphomas linked to hepatitis C virus infection and their microenvironment

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Abstract

The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent pattern; (2) a dependent pattern on deregulated interactions; and (3) a dependent pattern on regulated coexistence. Typical examples of the third pattern are hepatitis C virus (HCV)-associated marginal zone lymphomas (MZLs) and mucosa-associated lymphoid tissue lymphomas. In these lymphomas, a regulated coexistence of the malignant cells and the microenvironmental factors usually occurs. At least initially, however, tumor development and cell growth largely depend on external signals from the microenvironment, such as viral antigens, cytokines, and cell-cell interactions. The association between HCV infection and B-cell lymphomas is not completely defined, although this association has been demonstrated by epidemiological studies. MZL and diffuse large B-cell lymphoma are the histotypes most frequently associated with HCV infection. Many mechanisms have been proposed for explaining HCV-induced lymphomagenesis; antigenic stimulation by HCV seems to be fundamental in establishing B-cell expansion as observed in mixed cryoglobulinemia and in B-cell lymphomas. Recently, antiviral treatment has been proved to be effective in the treatment of HCV-associated indolent lymphomas. Importantly, clinically responses were linked to the eradication of the HCV-RNA, providing a strong argument in favor of a causative link between HCV and lymphoproliferation.

Key words: Hepatitis C virus-infection; B-cell lymphomas; Marginal zone lymphoma; Mucosa-associated lymphoid tissue lymphomas; Diffuse large B-cell lymphomas; Microenvironment

Core tip: The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent pattern; (2) a dependent pattern on deregulated interactions; and (3) a dependent pattern on regulated coexistence. The association between hepatitis C virus infection and B-cell lymphomas is not completely defined, although this association has been demonstrated by epidemiological studies.

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INTRODUCTION

Genetic alterations and abnormal microenvironmental factors are involved in tumor development, cell growth and disease progression. Inflammatory cells and soluble mediators, *i.e.*, cytokines and chemokines, are essential microenviromental factors that sustain cell growth and invasion, induce angiogenesis and suppress anti-tumor immune functions[1].

In multidimensional studies on hematolymphoid malignancies, a relevant clinical role of the tumor microenvironment has recently emerged, bringing new knowledge and suggesting new ideas and targets for treatment[2-5].

The relationships between lymphomas and their microenvironment appear to follow 3 major patterns: (1) an independent, largely autonomous pattern; (2) a dependent on deregulated interactions pattern; and (3) a dependent on regulated coexistence pattern[2]. A typical example of the first pattern is Burkitt lymphoma where all tumor cells proliferate because of permanent *MYC* gene activation. A typical example of the second pattern is classic Hodgkin lymphoma, where Reed-Sternberg cells escape the regulated cell growth- and proliferation-control. Typical examples of the third pattern are Hepatitis C virus (HCV)-associated marginal zone lymphomas (MZLs) and mucosa-associated lymphoid tissue (MALT) lymphomas. In this pattern, a regulated coexistence of the malignant cells and the microenvironment is reminiscent of the pattern that the normal counterpart B cells engage in with their respective microenvironment. At least initially, tumor development and cell growth largely depend on external signals from the microenvironment, such as viral antigens, cytokines, and cell-cell interactions[6].

HCV infection is a worldwide problem. There are important regional differences in the prevalence of HCV infection: the lowest rates are reported in Northern Europe while prevalence estimates exceed 2% in Italy, Japan, Egypt and southern parts of United States[7]. Among the carcinogenic viruses recognized by the recent International Agency for Research on Cancer (IARC) monograph Epstein-Barr virus (EBV), human papilloma virus (HPV), human T-lymphotropic virus type I (HTLV-1), and Kaposi sarcoma-associated herpesvirus (KSHV) play a direct role in carcinogenesis encoding oncoproteins which are able to promote cellular transformation[8,9]. Conversely, HCV and Helicobacter pylori appear to have an indirect role, by inducing a chronic inflammation[10]. Hepatitis B virus (HBV) has both a direct and indirect role in promoting hepatocellular carcinoma (HCC); as a matter of fact, chronic HBV carriers can develop HCC without developing cirrhosis.

PATHOGENETIC ASPECTS

HCV infection is the cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (Table 1). HCV infection has been also associated to a spectrum of extra-hepatic lymphoproliferative disorders including mixed cryoglobulinemia (MC)[11], the most well defined disorder associated with HCV infection, monoclonal gammopathies[12] and B-cell lymphomas[13]. HCV infection has been associated with B-cell low grade indolent lymphoma, especially of marginal zone origin, as well as with aggressive lymphomas, mainly diffuse large B-cell lymphomas (DLBCL) (Table 2). Authoritative studies have demonstrated that in HCV-infected patients with indolent lymphomas, eradication of HCV with antiviral treatment (AT) could directly induce lymphoma regression, providing a strong argument in favor of a causative link between HCV and lymphoproliferation[14].

INFLAMMATORY MICROENVIRONMENT

The liver is the main target of HCV infection and the major site of inflammatory events, including recruitment of inflammatory cells.

Occurrence of HCV enrichment in intrahepatic inflammatory infiltrates supports the notion that HCV is directly involved in the emergence and maintenance of these B-cell expansions[15]. Intrahepatic B-cell clonalities are invariably associated with extrahepatic manifestations of HCV infection associated B-cell lymphomas.

HCV AND LYMPHOMAGENESIS

According to the recent IARC monograph on biological agents and carcinogenesis, the association between HCV infection and B-cell lymphomas is not completely defined[9]. This association has been demonstrated by epidemiological studies in highly endemic geographical areas[16]. The role of HCV infection in lymphomagenesis may be related to the chronic antigenic stimulation of B-cell response, similar to the well characterized induction of gastric MALT lymphoma development by *Helicobacter pylori* chronic infection[17]. In fact, chronic HCV infection may sustain a multi-step evolution from MC to overt low grade lymphoma and eventually to high-grade lymphoma[17]. During this process, additional genetic aberrations may induce independence from antigenic stimulation. The clonal component of MC is often an IgM with a rheumatoid factor activity that mirrors the expansion of a B-cell monoclonal population not only in bone marrow but also in liver. It has also been suggested that HCV antigens (NS3 and E7or E2) can play a role in lymphomagenesis[18,19]. Recently, it has been published that HCV-related cryoglobulins (either IgM and IgG) are mainly directed against core and NS3 proteins[20]. Chromosomal alterations could also play a role in development of HCV-related lymphoproliferative disorders: for instance, MC with or without lymphoma is characterized by translocation t(14; 18) with the overexpression of the antiapoptotic *bcl-2* gene leading to prolonged B-cell survival[21].

Importantly, cytokines and chemokines (IFNγ, TNFα, CXCL13 and BAFF in MC[16], as well as osteopontin[22]) are involved in the mechanisms of HCV-

Table 1 Biological agents assessed by the International Agency for Research on Cancer Monographs Working Group[9]

Group-1 agent	Cancers on which sufficient evidence in humans is based	Other sites with limited evidence in humans	Established mechanistic events
Epstein–Barr virus	Nasopharyngeal carcinoma, Burkitt lymphoma, Immune-suppression-related non-Hodgkin lymphoma, Extranodal NK/T-cell lymphoma (nasal type), Hodgkin lymphoma	Gastric carcinoma[1] Lympho-epithelioma-like carcinoma[1]	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Hepatitis B virus	Hepatocellular carcinoma	Cholangiocarcinoma[1], Non-Hodgkin lymphoma[1]	Inflammation, liver cirrhosis, chronic hepatitis[2]
Hepatitis C virus	Hepatocellular carcinoma, Non-Hodgkin lymphoma[1]	Cholangiocarcinoma[1]	Inflammation, liver cirrhosis, liver fibrosis
Kaposi sarcoma herpes virus	Kaposi sarcoma[1], Primary effusion lymphoma[1]	Multicentric Castleman's disease[1]	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Human immunodeficiency virus, type 1	Kaposi sarcoma, Non-Hodgkin lymphoma, Hodgkin lymphoma[1], Cancer of the cervix[1], anus[1], conjunctiva[1]	Cancer of the vulva[1], vagina[1], penis[1], Non-melanoma skin cancer[1], Hepatocellular carcinoma[1]	Immunosuppression (indirect action)
Human papillomavirus type 16 (For the other types see Table 2)	Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx and tonsil	Cancer of the larynx	Immortalization, genomic instability, inhibition of DNA damage response, anti-apoptotic activity
Helicobacter pylori	Non-cardia gastric carcinoma, Low-grade B-cell mucosa-associated lymphoid tissue gastric lymphoma[1]		Inflammation, oxidative stress, altered cellular turnover, changes in gene expression, methylation, mutation

[1]Newly identified link between virus and cancer. In red are highlighted the lymphoid proliferations; [2]HBV has both a direct and indirect role in promoting hepatocellular carcinoma. Modified and adapted from Bouvard *et al*[8].

Table 2 Hepatitis C virus-associated indolent and aggressive lymphoid proliferations

Subtype	Variant	Specific lymphoma sites
Monoclonal B-cell lymphocytosis		
Tissue based monoclonal B cell and plasma cell proliferations of uncertain type		
Lymphoplasmocytic lymphoma/WM		
Chronic lymphocytic disorders (non CLL)		
MZL		
	Splenic MZL	
	Nodal MZL	
	MALT	Gastric
		Extranodal non gastric
		Salivary gland
		Skin
		Orbit
		Liver
Diffuse large B-cell lymphoma		

WM: Waldenström's macroglobulinemia; CLL: Chronic lymphocytic leukemia; MZL: Marginal zone lymphoma; MALT: Mucosa-associated lymphoid tissue.

induced lymphoproliferation.

HCV INFECTION AND SPECIFIC LYMPHOMAS

Within indolent lymphoma subtypes, the association with HCV infection has been best characterized in MZLs. Other infectious agents have been involved in the pathogenesis of specific types of MZLs. For examples Helicobacter pylori, Borrelia burgdorferi, and Chlamydophila psittaci have been involved in MALT lymphomas arising in stomach, skin and orbit, respectively[9]. Conversely, chronic stimulation by HCV plays a role in development of splenic marginal zone lymphoma (SMZL) and primary nodal marginal zone lymphoma. Primary nodal marginal zone lymphoma is a distinct clinical-pathological subtype characterized by exclusive primary lymph node localization in the absence of extranodal site of involvement (Figure 1).

Splenic and nodal MZL are indolent B-cell lymphomas corresponding to post-germinal center memory B cells that are supposed to derive from marginal zone[23,24]. These entities share some morphologic and pathogenic features, but have distinctive clinical presentation, immunophenotype and molecular abnormalities. Histologically, when the marginal zone B cells surround normal follicles with benign mantle zones as a third outer layer, they produce a marginal zone pattern[25]. As the marginal zone cells extend outwards into the interfollicular areas, they form confluent clusters resulting in an interfollicular pattern or a diffuse pattern in the absence of any follicles at later stages of the disease[25]. The marginal zone cells may also grow inwards into the follicles and produce either partial or complete follicular colonization[25] (Figure 1).

Gastric and non-gastric extranodal MZL are typically indolent diseases of middle and advanced age; disseminated disease is present in nearly one-third of cases. Interestingly, three specific MALT lymphoma sites showed an elevated prevalence of HCV infection: salivary glands, skin and orbit[26]. The association of HCV infection and salivary glands lymphoma has been clearly demonstrated[27].

Moreover, a study on B-cell lymphoma in patients with Sjögren's syndrome and HCV infection reported an

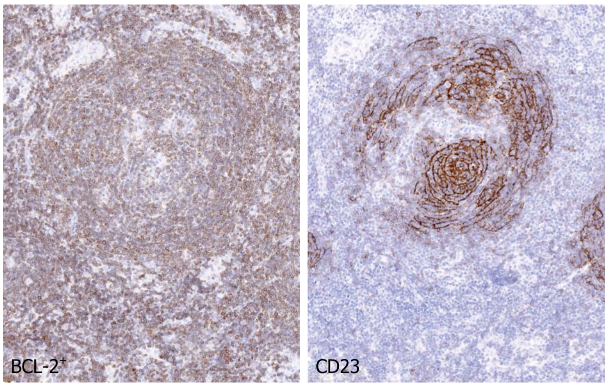

Figure 1 Nodal marginal zone lymphoma. The panel shows an example of nodal marginal zone lymphoma (MZL) with follicular colonization. BCL-2+ neoplastic cells surround and colonize the germinal center, whereas CD23 highlights the disrupted follicular dendritic cell meshwork. Images were acquired with the Olympus Dot. Slide Virtual microscopy system using an Olympus BX51 microscopy equipped with PLAN APO 2x/0.08 and UPLAN SApo 40x/0.95 objectives.

elevated occurrence of parotid involvement and a high proportion of MALT lymphomas with primary extranodal involvement (exocrine glands, liver, and stomach) (Table 2)[28].

Beside MZLs, also LPL/Waldenström's macroglobulinemia (WM) has been associated to HCV infection. However, this association is not completely defined. B-cell chronic lymphoproliferative disorders are defined as the miscellaneous category of lymproliferative disorders distinct from chronic lymphocytic. Association of these entities with HCV is not clear. Interestingly, monoclonal B-cell lymphocytosis (MBL), a pre-clinical condition characterized by an expansion of clonal B cells in the absence of frank lymphocytosis, was identified in nearly 30% of HCV-positive subjects with a significantly higher frequency than in the general population.

Recently, a retrospective study of B-cell lymphoproliferative disorders associated with HCV infection found two poorly described groups of cases. The first featured disseminated MZL without splenic MZL features, defying the current MZL classification; the other consisted of monoclonal B lymphocytes in the peripheral blood, bone marrow or other tissues, with no clinical or histological evidence of lymphoma. This pattern requires proper identification in order to avoid the misdiagnosis of the lymphoma[29].

Despite the classical association of HCV with indolent lymphoma, aggressive lymphoma, in particular DLBCL, are emerging as diseases linked to HCV infection. Patients affected by HCV-associated DLBCL display specific presentation with respect to HCV-negative DLBCL. In particular, residual signs of low-grade lymphoma and extranodal disease such as spleen are more frequently detected in HCV-associated DLBCL cases in comparison with HCV-negative DLBCL[9]. Unlike indolent B-cell lymphoma, AT seems not to play a central role in the first-line approach for HCV-associated DLBCL, because lymphoma cells are most likely to be independent from chronic antigenic stimulation due to the acquisition of additional oncogenic lesions. For this reason, HCV-associated DLBCL patients have to be treated with anthracycline-based chemotherapy coupled with rituximab.

CONCLUSION

Many epidemiological studies have provided evidence that HCV infection is associated with development of indolent and aggressive B-cell lymphoma[30,31]. However, the causal association between HCV infection and B-cell lymphomas is not completely defined. The similarities shared by rearranged Ig genes present in B cells from patients with type Ⅱ MC and malignant B-cells from HCV-positive patients affected by B-cell lymphoma support the possibility that the antigens that promote type Ⅱ MC and B-cell lymphoma in HCV-positive patients are the same[32,33]. These similarities also suggest that type Ⅱ MC may be a precursor of B-cell lymphoma[34]. Type Ⅱ MC probably plays a central role in the development of B-cell lymphoma in HCV-positive patients with Sjögren's syndrome[35].

Three hypothetic models have emerged to understand the molecular mechanisms of HCV-associated lymphoma development: (1) continuous external stimulation of lymphocyte receptors by viral antigens and consecutive proliferation; (2) direct role of HCV replication and expression in infected B-cells; and (3) permanent B-cell damage, *e.g.*, mutation of tumor suppressor genes, caused by a transiently intracellular virus ("hit and run" theory)[9,36]. Other non exclusive hypotheses have been proposed over the past two decades. These hypotheses have variously emphasized the important role played by chromosomal aberrations, cytokines, or microRNA molecules[37]. However, the mechanisms by which B-cell lymphomas are induced by HCV remain the subject of debate.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Burden of pediatric hepatitis C

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Abstract

Hepatitis C virus (HCV) is a major health burden infecting 170-210 million people worldwide. Additional 3-4 millions are newly-infected annually. Prevalence of pediatric infection varies from 0.05%-0.36% in the United States and Europe; up to 1.8%-5.8% in some developing countries. The highest prevalence occurs in Egypt, sub-Saharan Africa, Amazon basin and Mongolia. HCV has been present in some populations for several centuries, notably genotypes 1 and 2 in West Africa. Parenteral anti-schistosomal therapy practiced in the 1960s until the early 1980s had spread HCV infection throughout Egypt. Parenteral acquisition of HCV remains a major route for infection among Egyptian children. Insufficient screening of transfusions, unsterilized injection equipment and re-used needles and syringes continue to be major routes of HCV transmission in developing countries, whereas vertical transmission and adolescent high-risk behaviors (*e.g.*, injection drug abuse) are the major routes in developed countries. The risk of vertical transmission from an infected mother to her unborn/newborn infant is approximately 5%. Early stages of HCV infection in children do not lead to marked impairment in the quality of life nor to cognitive, behavioral or emotional dysfunction; however, caregiver stress and family system strain may occur. HCV slowly progresses to serious complications as cirrhosis (1%-2%) and hepatocellular carcinoma (HCC) especially in the presence of risk factors as hemolytic anemias, obesity, treated malignancy, and concomitant human immune deficiency and/or hepatitis B virus co-infection. HCV vaccine remains elusive to date. Understanding the immune mechanisms in patients who successfully cleared the infection is essential for vaccine development. The pediatric standard of care treatment consists of pegylated interferon-α 2a or b plus ribavirin for 24-48 wk. The new oral direct acting antivirals, approved for adults, need further evaluation in children. Sustained virologic response varies depending on the viral load, genotype, duration of infection, degree of aminotransferase elevation, adiposity and single nucleotide polymorphisms of interleukin (IL)-28B locus. The goals of treatment in individual patients are virus eradication, prevention of cirrhosis and HCC, and removing stigmatization; meanwhile the overall goal is decreasing the global burden of HCV. *IL-28B* polymorphisms have been also associated with spontaneous clearance of vertically acquired HCV infection. The worldwide economic burden of HCV for children, families and countries is estimated to be hundreds of millions of US dollars per year. The United States, alone, is estimated to spend 199-336 million dollars in screening, monitoring and treatment during one decade. The emotional burden of having an HCV infected child in a family is more difficult to estimate.

Key words: Hepatitis C virus; Burden; Genotypes; Cost; Pediatrics

Core tip: Hepatitis C virus (HCV) is a worldwide health burden infecting up to 5.8% of children in some developing countries with thousands of annual new

infections. HCV vaccine is illusive, but understanding immune mechanisms in patients who cleared infection may be crucial. The pediatric standard of care treatment is pegylated interferon-α2 plus ribavirin for 24-48 wk. The new oral direct acting antivirals need further evaluation in children. Interleukin-28B polymorphisms have been associated with treatment response and spontaneous clearance of vertical HCV infection. The worldwide economic burden of HCV is estimated to be hundreds of millions United States dollars/year. The emotional burden is difficult to estimate.

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INTRODUCTION AND EPIDEMIOLOGY

Hepatitis C virus (HCV) is a small, enveloped, positive-sense, single-stranded RNA virus of the *Flaviviridae* family[1]. HCV was first cloned in 1989 after more than 6 years of work to extract the virus from infected patients by a group of scientists from California in the United States[2]. HCV infection is recognized nowadays as a disease of global importance[3]. It is considered a major health and economic burden in adults as well as children in both developing and developed countries[3,4].

Viral hepatitis is the most common cause of liver disease in the world. Acute infections with their sequelae are responsible for 1-2 million deaths/year. Of them 54000 deaths are due to acute HCV infection[4]. After acute infection with HCV, as many as 50%-85% of patients fail to clear the virus resulting in chronic infection with 350000 deaths/year and 955000 disability due to related complications such as cirrhosis and liver cancer[5].

A recent systematic review found that globally between 1990 and 2005, the prevalence of people with anti-HCV has increased from 2.3% to 2.8%[4]. It is estimated that approximately 210 million individuals, *i.e.*, approximately 3% of the world population, are chronically infected with HCV[3,6] and 3-4 millions are newly infected each year[3]. Available data indicate that infection with HCV varies considerably by country and region, and the true burden of disease is not well known in many countries, because the capacity is often limited for collecting epidemiologic data[7]. The prevalence may vary markedly from one geographic area to another and even within the population assessed[8]. The highest prevalence of HCV is in Sub-Saharan Africa (5.3%), followed by the Eastern Mediterranean (4.6%), Western Pacific (3.9%) and South-Eastern Asia (2.15%) regions. Europe is thought to have the lowest prevalence of HCV (1.03%). In North America, prevalence is also low and estimated at 1.6% in the United States and 0.8% in Canada[9].

The study carried out by Uhanova *et al*[9] on the epidemiology of HCV in a North American population from the Canadian province of Manitoba, revealed several important findings: First, the diagnosis of HCV appears to have peaked in 1998 and has been relatively stable thereafter; second, the prevalence of HCV continued to increase amongst both men and women (4.6-fold during the 12-year period of the study). Overall, 84% of all subjects diagnosed since 1991 were alive in 2002, supporting the evidence of the growing burden of HCV; third, with the exception of young adults, males were 1.7 times more often infected than females; fourth, HCV infections were more common in urban centers.

Egypt has the highest worldwide prevalence with 9% countrywide rate; and up to 50% rates in certain rural areas due to specific modes of infection[10]. Prevalence in healthy Egyptian children is reported, by our group and others, to range from 1.4% to 5.8%[11,12]. Parenteral anti-schistosomal therapy, practiced in the 1960s until the early 1980s, had had a major role in the spread of HCV throughout Egypt[13].

GLOBAL HCV GENOTYPE DISTRIBUTION

By phylogenetic analysis, 6 distinct genotypes of HCV (denoted 1 to 6) and more than 100 subtypes (denoted 1a, 2c, 3d, 6f, *etc.*) have been described. Each genotype differs in its amino acid sequence by 31%-34%[14]. Genotypes 1-3 have a worldwide distribution, whereas 4 is found principally in Egypt, the Middle East and black Africa, 5 in South Africa, and 6 in Asia[15].

Genotype 1 (subtypes 1a and 1b) is by far the most prevalent genotype worldwide, with a higher prevalence of 1b in Europe and 1a in the United States. Genotype 3a is highly prevalent in European intravenous drug abusers[16]. This group is currently experiencing an increasing incidence and prevalence of infections related to HCV genotype 4 as well. Genotype 2 is found in clusters in the Mediterranean region[17]. Molecular clock analyses suggest that HCV strains have been present in some populations in their respective geographical regions for at least several centuries, notably genotypes 1 and 2 in West Africa and genotype 6 in Southeast Asia[18].

METHODS OF TRANSMISSION

Prior to the 1990s, the principal routes of HCV infection were via blood transfusion, unsafe injection procedures, and intravenous drug abuse. These modes of acquisition are estimated to account for approximately 70% of cases in industrialized countries. Epidemiological evidence shows that a wave of HCV infection occurred in the 1945-1965 period (baby boomers) in Western countries, as there was an increase in the use of injections, blood products and illicit drugs following World War II[1]. Screening of blood products for HCV by means of enzyme immunoassays and now, in an increasing number of countries, by nucleic acid testing (NAT) has virtually eradicated transfusion-transmitted HCV. Currently, new HCV infections are primarily due to intravenous or nasal drug abuse, and to a lesser degree to unsafe medical or

surgical procedures. Parenteral transmission via tattooing or acupuncture with unsafe materials is also implicated in occasional transmissions[8]. The risk of heterosexual transmission is low, while recent data indicate that promiscuous male homosexual activity is related to HCV infection[19].

In developing countries, insufficient screening of blood, blood products and parenteral exposure, continue to be the major causes of HCV transmission and are still reported among Egyptian children[20]. Unsafe use and reuse of injection equipment in hospitals is still a threat in many parts of Africa[21]. Intra-familial transmission may occur, but specific immune responses may be protective against house-hold infection in some children[22].

At present the vertical, maternal-neonatal or perinatal transmission is the most common route of pediatric HCV infection[23]. Worldwide, it has been estimated that 60000 HCV-infected infants are born yearly[24]. Mother-to-infant vertical transmission of HCV is reported to occur in approximately 5% of cases (with a range of 3%-10%), mostly in the late intrauterine period, at delivery or in the peri-natal period[1,24]. Many factors have been reported to influence the transmission rate[25], including maternal high viral load[26-28], labor duration, newborn gender, HCV genotype[29], human immuno-deficiency virus (HIV) co-infection[24], amniocentesis[30,31], fetal scalp monitoring[32], prolonged rupture of membranes[32,33] and fetal anoxia around the time of delivery[28].

The role of elective cesarean section to reduce mother-to-infant transmission rates is debated and controversial[33] and the guidelines of the European Association for the Study of the Liver (EASL) do not recommend cesarean section to prevent HCV vertical transmission[8]. Breast feeding is not considered to be contraindicated in women who are infected with HCV[34,35]. In spite that the majority of HCV-infected women do not transmit the virus to their offsprings, maternal uncertainty and guilt always surround possible transmission. Cost-effectiveness analysis based on available epidemiologic data indicates that screening of all pregnant mothers for HCV infection is not cost-effective[36]; however high risk mothers should be screened[25].

NATURAL HISTORY OF INFECTION

In some patients, HCV infection is a self-limited disease and HCV RNA becomes undetectable in most of these cases within 3 to 4 mo after the onset of acute infection[37]. Symptoms and signs following acute HCV infection are mild and usually non-specific; and fulminant HCV has not been reported in childhood[38].

Unfortunately spontaneous clearance of HCV occurs only in a minority of cases as 54%-86% of adult patients establish a chronic infection[39]. Many chronically infected patients do not know that they have been infected with HCV because infection is largely asymptomatic[40]. In the approximately 86% of infected patients who develop a chronic infection, HCV progresses insidiously with 10%-20% progressing to cirrhosis and approximately 7% of cirrhotic patients developing HCC[41].

Little is known about the characteristics of chronic HCV infection in children. Children rarely require liver transplantation for HCV infection. In the United States, only 133 children were transplanted for chronic HCV infection between 1988 and 2009[25]. To date, HCC is extremely uncommon in children with HCV infection[25]. Only 2 cases have been reported in children[42] and two further cases who had acquired chronic infection in childhood presented as young adults[43], however other unreported cases may exist. HCC complicating HCV infection may develop in the absence of cirrhosis[44,45], a finding of potential importance to pediatric patients[25]. Progression of liver affection depends on the viral load, serum aminotransferase levels, gender, ethnicity, obesity, toxins, environmental factors and co-morbid risk factors such as hemolytic anemias, treated malignancy, immunosuppression, and concomitant HIV or hepatitis B virus infection, or genetic factors *e.g.*, single-nucleotide polymorphisms (SNPs) of interleukin (IL)-28B gene locus[46].

Chronic HCV infection in children is associated with a variety of histological patterns of liver disease, generally not as severe as in adults. Indeed in many children, liver biopsy may disclose no obvious histological changes or only mild inflammation and fibrosis. Nevertheless, significant fibrosis or cirrhosis may occur[47]. Reports on the histological features and progression of hepatitis C in children are scarce but are generally milder than in adults[48]. In 1997, Kage *et al*[49] reported, in a cohort of Japanese children with chronic HCV infection, that liver histopathology presents the same lesions as in adults such as lymphoid aggregates, sinusoidal lymphocytosis and steatosis[48]. The stage of fibrosis in this cohort was mild, with only a 3.6% prevalence of bridging fibrosis with architectural distortion, and no cases of cirrhosis[48]. This seemingly mild course is in contrast with the findings in some of the earlier clinical reports in which the prevalence of cirrhosis was found to be up to 14%[50-52]. In a North American study carried out by Badizadegan *et al*[48], the characteristic histopathological lesions occurred with approximately the same frequencies in children as have been reported in adults. Necroinflammatory activity was generally mild. Portal fibrosis was present in 78% of the specimens, including fibrous portal expansion (26%), bridging fibrosis (22%), bridging fibrosis with architectural distortion (22%), and cirrhosis (8%). Centrilobular pericellular fibrosis, which has not been previously reported in the context of chronic HCV infection in adults or children, was also a prominent feature in their series, occurring with a similar frequency as steatosis or portal lymphoid aggregates/follicles. They suggested that in spite of mild histological necroinflammatory activity in general, the stage of fibrosis in children can be severe in spite of relatively short duration of infection[48].

EXTRAHEPATIC MANIFESTATIONS OF HCV

Chronic HCV infection may cause numerous extrahepat-

ic manifestations. Up to 40%-74% of patients with HCV infection develop at least one extrahepatic manifestation during their life time. The disorder with strongest link to HCV infection in adults is mixed cryoglobulinemia[46]. Other common symptoms are peripheral polyneuropathy, Raynaud's syndrome and sicca-like symptoms. Seven and a half to 10% of the patients develop a B-cell lymphoma at some point[53-55]. The clinically most relevant manifestation of mixed cryoglobulinemia is membrano-proliferative glomerulonephritis, which appears in 30%-36% of the cases and significantly increases mortality[54,55]. Membrano-proliferative glomerulonephritis may occur in children with chronic HCV infection, but unlike in adults, neither cryoglobulinemia nor lymphoma has yet been reported in children[25].

Another important extrahepatic manifestation of HCV infection is the involvement of the central nervous system. About 20%-80% of the patients with chronic HCV infection develop fatigue at some point independent from the severity of hepatitis. Fatigue often is the predominant complaint of the patients and might reduce the quality of life to a large extent. Patients may also develop depression or a general cognitive impairment irrespective of the stage of liver disease[56] which may be linked to HCV-induced neuro-inflammation and brain dysfunction[57]. These observations raise the issue of learning impairment in children with chronic HCV[25].

Impaired quality of life, potentially severe enough to have a negative effect on learning, has been reported in children with chronic HCV infection including developmental delay, learning disorders, and cognitive deficits less severe than those of attention deficit hyperactivity disorder but still reflecting decreased executive function[58,59].

COSTS OF INFECTION

Vietri *et al*[40] studied the burden of HCV in Europe and found that HCV patients compared to healthy controls have more impairment in work and non-work activities, and more annual physician visits per patient. Work-productivity impairment due to HCV costs over €7500 per employed patient per year[40]. Health-related quality of life was lower among HCV patients. Treatment-naïve HCV patients reported higher work impairment and more frequent physician visits. Each treatment-naïve HCV infected patient incurred €934 in direct costs. Employed treatment-naïve patients reported higher productivity loss per year[40]. In comparison, in the United States, Menzin and his group estimated that it costs $4956/patient in the year following the diagnosis of advanced liver disease secondary to HCV which were largely driven by inpatient costs[60].

There are no precise estimates of the true costs of HCV for a child and family. In one country like the United States, it is likely that several thousand children per year need treatment costing several thousand dollars/child; and it is estimated that in one decade, 26 million dollars will be spent in screening, 117-206 million dollars in monitoring, 56-104 millions dollars in treatment and the total cost would be about 199-336 million. Worldwide, global costs would be millions of dollars/year[61].

Treatment of a child which results in virus eradication is highly cost-effective because of the higher costs of the long-term consequences of untreated HCV cirrhosis and/or HCC. The small numbers of liver transplants for children with HCV performed each year cost several million dollars. The emotional costs of having an HCV infected child are more difficult to estimate for the child and family; but are real[61].

PREVENTION

The reduction of global morbidity and mortality related to chronic HCV infection should be a concern to public health authorities, and primary, secondary and tertiary prevention activities should be implemented and monitored in each country, with precise targets set to be reached. A working group was created to assist the World Health Organization in estimating the global burden of disease associated with HCV infection[3]. Public awareness of the transmission and prevention of HCV is crucial in decreasing the incidence and prevalence of the disease. Public and physician education in various forms is therefore extremely important. There is a need for implementing evidence-based international guidelines for preventing and managing hepatitis C in children worldwide[62].

One of the major hurdles in the eradication/reduction of the burden of HCV is the lack of hepatitis C vaccine. An effective HCV vaccine remains elusive to date. HCV has been difficult to target with a vaccine because it has many different strains. In addition, HCV mutates rapidly and exists as a complex family of mutated viruses within each infected individual (quasispecies) allowing the infecting virus to escape control by the immune system. This makes it difficult to identify which part of the virus should be targeted for developing a vaccine[62].

Viral and host specific factors contribute to viral evasion and present important impediments to vaccine development. Both, innate and adaptive immune responses are of major importance for the control of HCV infection. However, HCV has evolved ways of evading the host's immune response in order to establish persistent infection. For example, HCV inhibits intracellular interferon (IFN) signaling pathways, impairs the activation of dendritic cells, $CD8^+$ and $CD4^+$ T cell responses, induces a state of T-cell exhaustion and selects escape variants with mutations $CD8^+$ T cell epitopes[63]. An effective vaccine will need to produce strong and broadly cross-reactive $CD4^+$, $CD8^+$ T cell and neutralizing antibody (NAb) responses to be successful in preventing or clearing HCV. Vaccines in clinical trials now include recombinant proteins, synthetic peptides, virosome based vaccines, tarmogens, modified vaccinia Ankara based vaccines, and DNA based vaccines. Several pre-clinical vaccine strategies are also under development and include recombinant adenoviral vaccines, virus-like particles, and synthetic peptide

vaccines. Moreover, vaccines may also be used in the future in combination with the recent direct acting antiviral (DAA) drugs enabling IFN-free treatment regimens[63]. Indeed understanding the immune mechanisms, particularly HCV-specific cell mediated immune response, of patients who have successfully cleared the infection is essential to design and develop a vaccine[64].

Because there is no vaccine and no post-exposure prophylaxis for HCV, the focus of primary prevention efforts should be safer blood supply in the developing world, safe injection practices in health care and other settings, and decreasing the number of people who initiate injection drug abuse[6]. People with known HCV infection should be counseled regarding ways to reduce the risk of transmitting HCV to others, and means of minimizing their risk for HCV-related complications. As part of secondary prevention efforts, HCV-infected people should be referred for medical evaluation and antiviral treatment consideration, and programs ensuring access to these services should be in place[6].

Health education is also essential to reduce the HCV burden, and specific programs should be provided to increase public awareness on transmission and prevention of infection[62].

TREATMENT

HCV is a potentially curable disease[65] with a good percentage of treated patients getting a sustained virologic response (SVR) defined as undetectable serum HCV RNA 24 wk after the end of therapy (and now at 12 wk after the end of therapy[8]). Although the available standard of care (SOC) therapy has led to significant improvements in treatment response rates, less than 50% of HCV-infected persons are aware of their diagnosis[66], and among them, only 1%-30% receive treatment. The true rate-limiting factor in achieving better outcomes may turn out to be access to diagnosis and treatment[66].

Multiple barriers may impede the delivery of HCV therapy[67]. To increase cure rates, the psychological (psychiatric illness, attitudes and coping skills), lifestyle (alcohol consumption, diet, and exercise), social (income, education, social class, poverty), and other different barriers to treatment adherence and completion must be identified and overcome[68].

For adults, standard IFN has been approved for the treatment of HCV since 1991, Ribavirin (RBV) since 1998 and pegylated-IFN (peg-IFN) since 2002. Nine years had passed before the American Food and Drug Administration (FDA) approved a new drug to be added to the excising SOC, the DAA oral protease inhibitors, boceprevir and telaprevir[69]. New drugs under development include other protease inhibitors, the NS5B polymerase and NS5A inhibitors[70]. In the coming years, the number of the new drugs will multiply exponentially and pharmaceutical companies have begun to combine them in triple and quadruple regimens (with and without peg-IFN)[69].

In children 3-17 years old, treatment with peg-IFNα-2 a or b plus RBV for 24-48 wk is the SOC therapy[71,72], whereas the recently approved DAA still need evaluation in children.

In the United States and most European countries, the current first line therapy for infection with genotype-1 is a combination of peg-IFN alpha plus RBV plus either boceprevir or telaprevir. High SVR rates can be achieved even in those with evidence of fibrosis and cirrhosis, but response is poor in prior null responders, especially those with cirrhosis[73].

Preliminary data from investigational studies suggest the potential for cure rates of 80%-90% in genotype-1 infection using combinations of DAAs and RBV without peg-IFN, but larger studies will be needed to confirm these results across a wider range of populations[74-76].

Quadruple therapy with pegylated-IFN combines BI201335, a protease inhibitor, and BI207127, a non-nucleoside NS5B polymerase inhibitor, with peg-IFN and RBV[77]. The only quadruple peg-IFN-free study is the Gilead Sciences all-oral quad regimen[78]. It is a phase II study for genotype 1, treatment-naïve patients who are not cirrhotic. It combines GS-5885, GS-9451, tegobuvir, and RBV for 24 wk. The triple peg-IFN-free therapy combines mericitabine, a nucleoside NS5B polymerase inhibitor; danoprevir, a protease inhibitor; ritonavir, a booster for danoprevir; and RBV or placebo[79].

SVR varies considerably from 26%-80% depending on age, duration of infection, viral load, viral genotype, adiposity, hepatic fibrosis iron scores, aminotransferase elevation, compliance with therapy, SNPs of IL-28B gene locus. It was found that a single *IL28B* genotype SNP rs12979860 determination predicts treatment response in patients with chronic hepatitis C Genotype 1 virus[80,81]. IL-28B has been also reported to play a role in spontaneous clearance of HCV genotype 4 in Egypt/North Africa[82]. Regarding the HCV genotypes 2 and 3, the polymorphisms rs12979860 and rs8099917 showed significant associations. However, the strength of this association was almost three times lower than for genotypes 1 and 4[83]. In addition regarding genotype 2, it was found that the Asian population was solely responsible for this association in rs8099917[84]. The generally reduced association for patients with HCV genotypes 2/3 could be related to the high rate of SVR present in these IFN-sensitive genotypes[85].

SNPs of *IL-28B* gene received considerable interest also for their association with spontaneous clearance of HCV among vertically-infected children[86].

SNPs of *IL-28B* as well as *IL-10* are good predictors of response to IFN/RBV therapy in HCV genotype 4 infected Egyptian children[87].

The combination of serum level of IFN-gamma inducible protein and SNPs of IL-28B can identify patients with acute HCV who are most likely to undergo spontaneous clearance and those in need of early antiviral therapy[88].

SNPs of osteopontin gene were also reported as pre-

dictors for the efficacy of IFN therapy in chronic HCV Egyptian patients with genotype 4[89].

CONCLUSION

HCV infection is an increasing health and economic burden in adults as well as children, in both developing and developed countries. The natural history and histopathology of HCV-related liver disease in children are still conflicting and variable. Prevention of infection depends on screening of blood with the most sensitive tests, avoiding nosocomial infections, and avoiding injection drug abuse and unprotected sex in adolescents; as well as education. Development of a vaccine preventing HCV infection is of thorough public health importance. The SOC therapy is peg-IFN plus RBV. The SVR is variable (26%-80%) and depends on several viral and host factors. Eradication of HCV in a child (if possible) is cost-effective as it may prevent cirrhosis and HCC; and can have major family, public health and global benefits.

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TOPIC HIGHLIGHT

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Direct effects of hepatitis C virus on the lymphoid cells

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Abstract

It has been reported that the direct binding of hepatitis C virus (HCV) and/or the replication of HCV in the extrahepatic organs and, especially, lymphoid cells, might affect the pathogenesis of extrahepatic diseases with HCV infection. More than one decade ago, several reports described the existence of HCV-RNA in peripheral blood mononuclear cells. Moreover, many reports describing the existence of HCV in B lymphocytes and B cell lymphoma have been published. In addition to B lymphocytes, it was reported that HCV replication could be detected in T lymphocytes and T cell lines. Among the extrahepatic diseases with HCV infection, mixed cryoglobulinemia-related diseases and autoimmune-related diseases are important for understanding the immunopathogensis of HCV persistent infection. Moreover, HCV persistent infection can cause malignant lymphoma. The biological significance of lymphotropic HCV has not yet become clear. However, several candidates have been considered for a long time. One is that lymphotropic HCV is an HCV reservoir that might contribute to the recurrence of HCV infection and difficult-to-treat disease status. The other important issue is the carcinogenesis of the lymphoid cells and disturbances of the immune responses. Therefore, the extrahepatic diseases might be induced by direct interaction between HCV and lymphoid cells. In this article, we summarize various studies showing the direct effect of HCV on lymphoid cells and discuss the biological significance of lymphotropic HCV.

Key words: Hepatitis C virus; Lymphotropism; T cell; B cell; Immunology

Core tip: In this article, we summarize various studies showing the direct effect of hepatitis C virus (HCV) on lymphoid cells and discuss the biological significance of lymphotropic HCV.

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INTRODUCTION

An estimated 130-170 million people are infected with hepatitis C virus (HCV) worldwide[1]. Around 75% of the patients with acute HCV infection undergo chronic HCV infection and are subsequently at risk of progressing to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[1,2]. Persistent infection of HCV involves not only the liver but also various extra-hepatic organs[3-7]. HCV can infect hepatocytes, lymphoid cells, and probably other cells through CD81 and receptor candidates[8]. Moreover, the expression of microRNA (miR)-122 facilitates efficient replication of HCV in nonhepatic cells[9]. These reports indicated that the direct binding of HCV and/or the replication of HCV in the extrahepatic organs, especially lymphoid cells, might affect the pathogenesis of

extrahepatic diseases with HCV infection. Among the extrahepatic diseases with HCV infection, mixed cryoglobulinemia (MC)-related diseases and autoimmune-related diseases are important for understanding the immunopathogenesis of HCV persistent infection[10-13]. Moreover, HCV persistent infection could cause malignant lymphoma[4]. The status of a disease might depend on the direct interaction between HCV and lymphoid cells[6,14-17]. The biological significance of lymphotropic HCV has not yet become clear. However, several candidates have been considered for a long time. One is that lymphotropic HCV is an HCV reservoir that might contribute to the recurrence of HCV infection and difficult-to-treat disease status[18-23]. The other important issue is the carcinogenesis of the lymphoid cells and disturbances of the immune responses[8,14,24-28]. Previously, Sung *et al*[29] reported a lymphotropic HCV strain that was isolated from B cell lymphoma. This lymphotropic HCV strain can infect and replicate in established B cell lines and primary B lymphocytes[29]. Moreover, we reported that T cell lines and primary naïve T lymphocytes were infected with this HCV strain[8,25,26]. In these studies, we demonstrated that lymphotropic HCV had various effects, especially on T cell development and proliferation. Therefore, understanding of the direct effects of HCV on the lymphoid cells is needed to clarify the immunopathogenesis of HCV persistent infection. In this report, we summarize various studies showing the direct effect of HCV on lymphoid cells and discuss the biological significance of lymphotropic HCV.

ROLE OF VIRUS RESERVOIR

HCV infection in peripheral blood mononucleated cells

More than one decade ago, several reports described the existence of HCV-RNA in peripheral blood mononucleated cells (PBMCs)[30,31]. The detection rate of HCV-RNA in PBMCs was increased if the patients were infected with human immunodeficiency virus (HIV) and HCV[31]. This phenomenon indicated that immune-suppressive circumstances and/or HIV antigen might enhance the replication activity of HCV in lymphoid cells[32]. HIV-1 accessory protein transactivator of transcription (TAT) can activate HCV replication by upregulating IP10 production. Moreover, it was reported that continuous release of HCV by PBMCs was detected in HCV-infected patients, especially in HIV co-infected patients[18]. The detection of HCV-RNA in the PBMCs from HIV-HCV co-infected patients could contribute to the recurrence of HCV viremia after pegylated-interferon and ribavirin treatment. It was reported that the presence of positive/negative strand HCV RNA at the end of treatment is associated with relapse among HCV-HIV co-infected patients[33]. In addition to HCV-HIV co-infected patients, a low level of HCV replication could be detected in peripheral lymphoid cells from HCV mono-infected patients after antiviral treatment[20,23]. Moreover, it was reported that HCV persisting at low levels long after therapy-induced resolution of chronic hepatitis C could remain infectious[20]. This continuous viral presence could result in the persistence of humoral and cellular immunity for many years after treatment and could present a risk of infection reactivation.

Responsible lymphocyte subsets as a viral reservoir

It has been reported that HCV replication could be detected in various kinds of lymphoid cells. Many reports describing the existence of HCV in B lymphocytes and B cell lymphoma have been published[5,29,34]. Recently, one group reported that $CD19^+$ B lymphocytes had significantly higher viral loads than $CD14^+$ monocytes[35]. Among B lymphocytes, $CD27^+$ memory B lymphocytes were more resistant to apoptosis than $CD27^-$ B lymphocytes. $CD27^+$ B lymphocytes might be a candidate subset of the HCV reservoir in chronic hepatitis C (CH-C)[36]. In addition to B lymphocytes, it was reported that HCV replication could be detected in T lymphocytes and T cell lines[20,37,38]. We also reported that a lymphotropic HCV strain could infect T cell lines and primary human naïve $CD4^+$ T lymphocytes[8,25,26]. HCV infects hepatocytes, lymphoid cells, and probably other cells through CD81 and several candidate receptors. The expression of CD81 could be detected in B cells, T cells, and monocytes, indicating that these types of cells are potential targets of HCV infection. Recently, one group reported that HCV infection of human T lymphocytes is mediated by CD5[39]. In contrast to T lymphocytes, hepatocytes do not express CD5. Therefore, the mechanism of HCV lymphotropism might be different from that of HCV hepatotropism. Moreover, the other candidate receptors were analyzed using HCV-prone and resistant T cell lines, PBMCs, primary T cells, Huh7.5 cells and HepG2 cells[40]. CD5 and CD81 expression coincided with lymphotropism and that of occludin with the permissiveness of T cell lines, but probably not primary T lymphocytes[40].

In addition to B and T lymphocytes, it has been reported that HCV can infect monocytes, especially $CD14^+CD16^+$ monocytes, but not $CD14^+CD16^-$ monocytes[41]. The detection of HCV-RNA in monocytes was reported in HCV-HIV co-infected patients and HCV-monoinfected patients[19]. HIV might facilitate the infection/replication of HCV in human macrophages[42]. One group reported the frequent compartmentalization of HCV in circulating $CD19^+$ B lymphocytes and $CD14^+$ monocytes[43]. Moreover, it was reported that immature and mature dendritic cells are susceptible to HCV genotype 1 infection, supporting at least HCV RNA replication *in vitro*[44]. Another group reported that replicative-strand HCV-RNA was detected in peripheral blood dendritic cells[28]. Although other lymphoid cells might be susceptible to HCV infection[45,46], these reports suggested that B and T lymphocytes, monocytes, and dendritic cells could be reservoirs for HCV.

DIRECT EFFECT OF HCV ON THE CARCINOGENESIS OF LYMPHOID CELLS

Many reports have focused on the relevance of HCV infection and B-cell lymphoma, especially non-Hodgkin lymphoma (NHL)[4,47]. Compared to the high association between HCV infection and HCC, epidemiologic reports on the relationship between HCV and NHL show a moderate risk for the development of lymphoma. However, no association between HCV and NHL was also reported in low HCV prevalence countries[48,49]. Different hypotheses have been suggested to explain the difference in the HCV-NHL prevalence: (1) Geographic differences in the HCV genotype distribution might contribute to differences in the HCV-NHL prevalence; (2) The duration of persistent infection of HCV might influence the carcinogenesis of lymphoid cells; and (3) Studies in low prevalence countries might not have included enough patients to detect the association. However, meta-analyses indicated a significant association between HCV and B-NHL[48,50].

Many groups reported the mechanisms of lymphomagenesis. However, we have to understand that HCV-infected patients with MC are at a higher risk of developing HCV-NHL[51]. MC could be an intermediary step in the development of NHL. Although, different theories have been proposed to explain the mechanism of HCV-induced lymphomagenesis, we can classify most of the theories into two categories. One of them is direct HCV binding with B lymphocytes. The external stimulation of lymphocyte receptors (CD19, CD21, CD81, B-cell receptor) by HCV antigen might induce a proliferation signal[52]. The HCV-core protein induces the production of interleukin (IL) 6 in CD14$^+$ cells *via* Toll like receptor 2 and leads to increased B cell proliferation[53]. In addition to the classical cytokine proliferation signal, the down regulation of miR-26b, an miRNA known to have tumor-suppressive properties, was found in splenic marginal zone lymphoma with HCV persistent infection[54]. This theory was supported by the phenomenon of lymphoma remission when the HCV antigens are removed by treatment. In addition to the proliferation signal, HCV-E2 CD81 on B cells triggers the enhanced expression of activation-induced cytidine deaminase (AID), which could contribute to enhancing the mutation frequency[14]. The other category of lymphomagenesis mechanism is HCV infection and/or replication in B lymphocytes. It has been reported that the replication of HCV in B lymphocytes could induce error-prone DNA polymerase zeta, polymerase iota, and AID, which contribute to enhancing the mutation frequency[14]. Moreover, the cellular DNA damage and mutation were mediated by nitric oxide and reactive oxygen species[55,56]. In addition to *in vitro* study, interferon regulatory factor-1-null mice with inducible and persistent expression of HCV structural protein showed a high incidence of lymphoma and lymphoproliferative diseases[57]. In this mouse model, the overexpression of apoptotic related genes and aberrant cytokine production were detected in the first step of carcinogenesis. Another group also reported that the expression of HCV-core protein could increase the incidence of lymphoma in transgenic mice[58]. Moreover, it has been reported that persistent expression of the full genome of HCV in B cells induces the spontaneous development of B-cell lymphoma *in vivo*[59]. HCV transgenic mice that expressed the full HCV genome in B cells showed a 25% incidence of diffuse, large B-cell non-Hodgkin lymphomas. Although the relationship between HCV persistent infection and lymphomagenesis could become clarified by various epidemiological studies, the mechanism of lymphomagenesis still needs to be considered carefully.

DIRECT EFFECT OF HCV ON THE IMMUNE EVASION

Many studies have described a failure of the innate and cellular immune response, including type 1 helper T cells (Th1) hypo-responsiveness, cytotoxic T lymphocytes (CTL) exhaustion, excessive function of CD4$^+$CD25$^+$ FOXP3$^+$ regulatory T cells, failure of dendritic cell function, occurs in HCV persistent infection[60-69]. Among the numerous mechanisms, the lymphoid cells, *via* direct binding and/or infection in B cells, T cells, NK cells and DCs *etc*., should be considered, especially in HCV persistent infection[8,25-28,70-73]. In our previous study, we used SB-cell lines that continuously produce infectious HCV virions in culture. The virus particles produced from the culture had a buoyant density of 1.13-1.15 g/mL in sucrose and could infect primary human PBMCs and an established B-cell line *in vitro*[29]. This lymphotropic HCV strain was useful to investigate the biological significance of HCV replication in lymphoid cells. In this *in vitro* system, HCV could infect and transiently replicate in T cells and HCV replication suppressed the interferon (IFN)-γ/STAT-1/T-bet signaling due to the reduction of STAT-1 and inhibition of its activation[26]. Moreover, HCV replication in T cells suppressed cellular proliferation and enhanced susceptibility to Fas signaling by inhibiting CD44v6 signaling and expression[25]. In addition to cell lines, we used primary T lymphocytes to analyze the biological meaning of lymphotropic HCV[8]. Another group reported that HCV core protein modulates the transcription of *IL-2* promoter in T lymphocytes by activating the nuclear factor of activated T lymphocyte pathway[74,75]. Moreover, the expression of HCV core protein could induce Ca^{2+} oscillations that regulate both the efficacy and information content of Ca^{2+} signals[74]. In addition to HCV replication in T cells, Yao *et al*[76] reported that the direct binding of HCV core to gC1qR on CD4$^+$ and CD8$^+$ T cells leads to impaired activation of Lck and Akt. We could also detect a relationship between HCV core protein and immune suppression in HCV persistent infection[77]. Double filtration plasmapheresis for CH-C patients could reduce the amounts of HCV core proteins in the peripheral blood and on the surface of T lymphocytes[77]. Moreover, it has been reported that the

engagement of gC1qR on DCs by HCV core limits the induction of Th1 responses and may contribute to viral persistence. Another group reported that NK cell-derived cytokines secreted in the presence of HCV cc showed a diminished antiviral effect that correlated with a reduction of IFN-γ[72]. DCs play essential roles in the triggering of primary antiviral immune reactions. DCs are the most potent activators of CD4 T cells for supporting Th1 differentiation, which is important for the cellular immune response. Several reports described that persistent HCV infection is associated with an allostimulatory defect of monocyte-derived DC[67,70]. These reports supported that HIV/HCV co-infected patients were difficult-to-control in comparison with HCV mono-infected patients, since lymphotropic HCV is frequently detected in HIV/HCV co-infected patients[78]. Co-infection with HCV and HIV is associated with increased HCV replication and a more rapid progression to severe liver disease, including the development of cirrhosis and HCC.

DIRECT EFFECT OF HCV ON IMMUNE STIMULATION

We need to focus not only on the suppression of the immune system but also on the stimulation of the immune system, since the prevalence of cryoglobuline-related and autoimmune-related diseases is much higher than in healthy subjects[10,79]. HCV core protein activates interleukin-2 gene transcription through the nuclear factors of activated T cells pathway[75,80]. IL-2 has a role in T cell proliferation. Recently, we reported that lymphotropic HCV and high frequency of Th17 cells were detected in CH-C patients with pyoderma gangrenosum-like lesions[16]. In that report, the eradication of HCV could improve the immunological status and pyoderma gangrenosum-like lesions. A study regarding the relationship between lymphotropic HCV and autoimmune diseases is ongoing in our laboratory. Another group reported that HCV-core induced STAT3 activation might play a role in the alteration of inflammatory responses in human monocytes[81]. Moreover, HCV infection of macrophage/monocytes *in vitro* might be associated with the induction of cytokines tumor growth factor-α and IL8. In addition to T lymphocytes and monocytes, Machida *et al*[17,27] reported that HCV could induce immunoglobulin hypermutation in B lymphocytes. These reports together suggest that HCV could stimulate an unfavorable immune response. As for the understanding of autoimmune diseases, HCV persistent infection might be one of the representative models of viral-induced autoimmune diseases.

CONCLUSION

Although various reports have described the direct effects of HCV on lymphoid cells, few have addressed whether the disturbance of the immune system induced by the direct binding and/or infection of HCV on lymphoid cells might coordinately influence the pathogenesis of HCV persistent infection. In this article, we summarized various reports indicating the direct effects of HCV on lymphoid cells. In addition to the direct effect of HCV, the indirect effects of HCV on lymphoid cells could influence the pathogenesis of HCV persistent infection. Therefore, we must treat a vast array of data to clarify the real pathogenesis of HCV persistent infection. Recently, the technologies of deep sequencing, immunoassays with increased numbers of multicolor flow cytometry analyses, and chimera mice with human lymphocytes have been developed. These technologies, together with previous data, might be able to clarify the direct effects of HCV on lymphoid cells.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

An insight into the diagnosis and pathogenesis of hepatitis C virus infection

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Abstract

This review focuses on research findings in the area of diagnosis and pathogenesis of hepatitis C virus (HCV) infection over the last few decades. The information based on published literature provides an update on these two aspects of HCV. HCV infection, previously called blood transmitted non-A, non-B infection, is prevalent globally and poses a serious public health problem worldwide. The diagnosis of HCV infection has evolved from serodetection of non-specific and low avidity anti-HCV antibodies to detection of viral nucleic acid in serum using the polymerase chain reaction (PCR) technique. Current PCR assays detect viral nucleic acid with high accuracy and the exact copy number of viral particles. Moreover, multiplex assays using real-time PCR are available for identification of HCV-genotypes and their isotypes. In contrast to previous methods, the newly developed assays are not only fast and economic, but also resolve the problem of the window period as well as differentiate present from past infection. HCV is a non-cytopathic virus, thus, its pathogenesis is regulated by host immunity and metabolic changes including oxidative stress, insulin resistance and hepatic steatosis. Both innate and adaptive immunity play an important role in HCV pathogenesis. Cytotoxic lymphocytes demonstrate crucial activity during viral eradication or viral persistence and are influenced by viral proteins, HCV-quasispecies and several metabolic factors regulating liver metabolism. HCV pathogenesis is a very complex phenomenon and requires further study to determine the other factors involved.

Key words: Hepatitis C virus; Diagnosis; Pathogenesis; Immunity; Steatosis

Core tip: This article focuses on the diagnosis and pathogenesis of hepatitis C virus infection. Both of these aspects are important in order to eradicate this endemic virus and to prevent serious liver diseases.

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INTRODUCTION

Hepatitis C virus (HCV) was first characterized by Choo *et al*[1] and Kuo *et al*[2] in 1989. It was soon identified as the main causative agent of the disease previously known as post transfusion non-A, non-B hepatitis virus infec-

tion. HCV has been found to be an important cause of liver disease and remains a major public health problem worldwide. According to the World Health Organization, nearly 3% of the world population has been infected with HCV. Therefore, more than 170 million people are chronic carriers of HCV and at high risk of developing liver cirrhosis and/or hepatocellular carcinoma (HCC). Three to 4% of chronically infected individuals develop fatal HCC. Currently, HCC caused by HCV infection is considered an indication for liver transplantation[3-5].

HCV was the leading cause of post-transfusion and community-acquired non-A, non-B hepatitis until characterization of the virus in 1989 and the introduction of blood screening in 1990. The initiation of blood screening for HCV has markedly reduced its incidence. However, it still remains a significant problem in intravenous drug abusers. HCV infection is the most common cause of liver transplantation in adults. HCV and HIV-1 frequently co-infect humans and it has been estimated that as many as 18% of HIV-infected persons are also infected with HCV[4].

HCV is an enveloped RNA virus and belongs to the genus Hepacivirus of the family Flaviviridae. The HCV genome consists of 9.6-kb single-stranded RNA of positive polarity and a single open reading frame of 9033-9099 nucleotides flanked by a conserved 5' and 3' noncoding region (NCR) at the ends. Its genome codes for a long polyprotein of approximately 3000 amino acids[6] which is processed co-translationally and post-translationally to yield structural proteins (core, envelope E1, and E2) and non-structural (NS) proteins (NS1/p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)[7]. The envelope proteins (E1 and E2) are the outer surface proteins of the viral particles and play important roles in virus entry into the host cell. NS5B is a variable region of the HCV genome and codes for an RNA-dependent RNA polymerase (RdRp).

RNA polymerase lacks proof reading activity and this may alter the detection, sensitivity to interferon anti-viral activity and pathogenicity of the virus (Figure 1)[8].

Like several other viruses, the RNA virus has a high degree of heterogeneity[5] that varies 30%-35% among different genotypes. Based on previous studies, six major genotypes and more than 120 subtypes of HCV have been characterized to date[9]. These HCV genotypes have distinct geographic distributions, with genotype 1 and 2 frequently found worldwide[10]. In India, genotype 3 is reported to be the most prevalent, followed by genotype 1[11,12]. Different HCV genotypes have important epidemiological implications. Despite nucleotide sequence divergence between genotypes, they remain quite similar in their transmission pattern, persistence and disease development[13]. Although genetic variation is attributed to several factors, two major theories *i.e.*, the Darwinian and Neutral evolution theories are thought to be the prominent theories in causing genetic diversity in HCV[13]. The nucleotide sequence variability is distributed throughout the viral genome. Regions encoding envelope proteins (E1, E2) and NS-1 are the most variable, whereas the 5' NCR is the most conserved region.

HCV patients show a poor response to antiviral therapy based on the combination of pegylated interferon (IFN)-α and ribavirin. Only 40%-50% of patients infected with HCV genotype-1 and 80% of those infected with genotype-2 or 3 achieve a sustained virological response (SVR) with this regimen[14]. The recent use of direct acting anti-viral (DAA) molecules, which are active on HCV during treatment, has led to a substantial improvement in SVR rates in HCV genotype-1 infected patients. However, this may lead to the selection of resistant virus if DAA molecules are used alone[15]. Moreover, there is a high relapse rate of HCV infection after discontinuation of therapy. Recently, host genetic factors including human leukocyte antigen (HLA) and cytokine genes have been implicated in HCV infection or persistence[16]. Genetic polymorphism of cytokine genes including *IFN*-γ, tumor necrosis factor (*TNF*)-α, interleukin (*IL*)-*10*, *IL-20* and SNPs in the promoter region of osteopontin gene, have been found to be crucial in determining the therapeutic outcome of HCV infection[17]. Therefore, every effort is being made to understand the pathogenesis of HCV infection to create a therapeutic model for an effective treatment against HCV. Although recent reports describe the development of *in vitro* replication systems leading to the production of infectious viral particles[18,19], there is currently no cell culture model suitable for synthesizing vaccines based on killed or attenuated virus. All efforts have been focused on sub-unit vaccines, composed of one or several antigens, either in the form of recombinant proteins, synthetic peptides or vectored vaccines. The earliest vaccine developed for HCV was that by the Chiron group[20]. However, very little progress was noted in this direction in subsequent years.

This article reviews the major aspects of HCV infection including the diagnosis and pathogenesis of HCV infection. Both of these aspects have a strong association with therapy, thus, newer means of accurate diagnosis and a better understanding of HCV infection pathogenesis may allow the development of a therapeutic model. This article attempts to update readers regarding the information available on these two aspects to date.

DIAGNOSIS OF HCV INFECTION

During HCV infection, every attempt is made to diagnose and differentiate acute from chronic hepatitis C infection. Acute HCV infection is typically mild. It is often not diagnosed, and the infection may be recognized only when it becomes chronic[21]. The diagnostic tests used, including the presence of anti-HCV antibodies in serum, cannot differentiate between acute and chronic HCV infection because anti-HCV IgM, used as marker of acute infection, is variable in acute infectious disease and is also detected at high rates in patients with chronic HCV infection[22,23]. The diagnostic procedures for hepatitis C virus infection used in laboratories are based on the detection

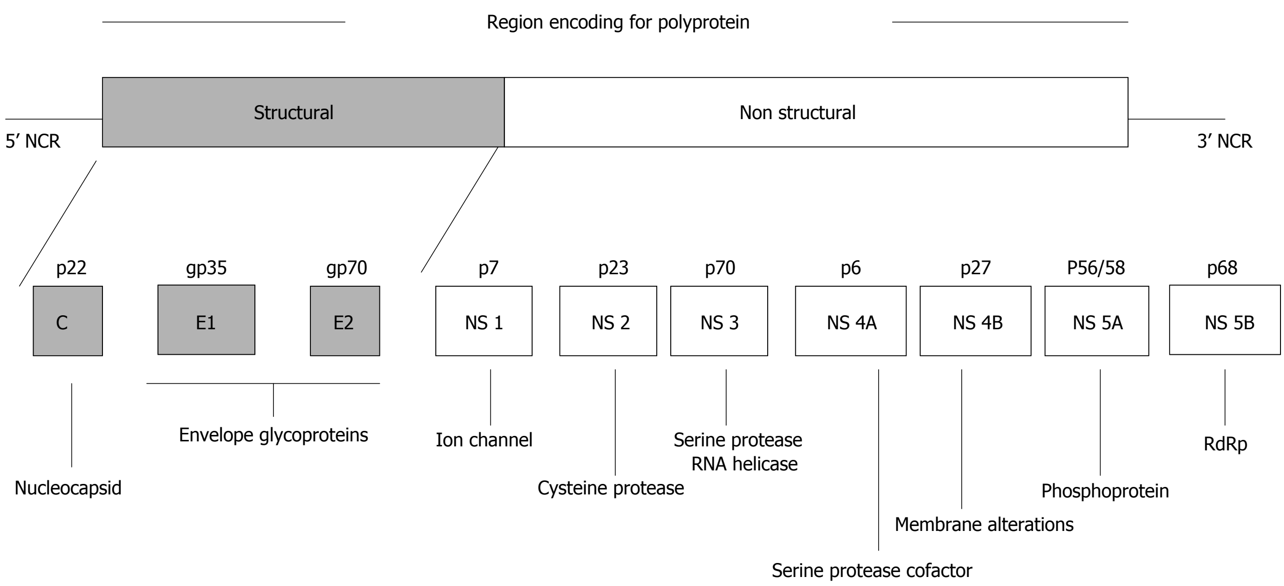

Figure 1 Proteins encoded by the hepatitis C virus genome. Genome organization of hepatitis C virus showing the structure of the viral genome, including the long open reading frame encoding structural and nonstructural proteins, and 5' and 3' non-coding regions (NCRs). [Source: Monica A *et al*. *Expert Rev Mol Med* 2003; 5].

of anti-HCV antibodies against recombinant HCV proteins using enzyme immunoassay (EIA) and chemiluminescence immunoassay. Non-structural and recombinant antigens are used in these assays. Four different generations of anti-HCV test kits have been developed to date. The first generation EIA detected antibodies against the nonstructural proteins (NS4) with recombinant antigen c100-3. Subsequently, the second generation assay was developed and this included antigens from the core region (c22-3), the NS3 region (c33c) and a part of c100-3 (5-1-1) from the NS4 region. The third-generation EIA included an additional antigen from the NS5 region and a reconfiguration of the core and NS3 antigens. However, all these anti-HCV assays had the disadvantages of giving high false positive results and a lack of sensitivity to detect antibodies during the window period. In addition, these antibody-based assays could not distinguish between acute, past and chronic infections. This was followed by the development of supplementary tests involving the recombinant immunoblot assay (RIBA) which was commercialized. This assay contained recombinant antigen (c33c, NS5) and synthetic peptides (5-1-1, c100 and c22). Similarly, a few other commercial assays, known as third generation immunoassays incorporated HCV antigens from the core region, E2 hypervariable region, NS3 region, NS4A, NS4B and NS5A region. All these recombinant immunoblot assays were used as supplementary tests to the anti-HCV assays. Similar to EIA, the RIBA had the disadvantages of difficulty in performance and a high percentage of indeterminate results. Therefore, these are no longer used in diagnostic laboratories. Recently, fourth generation anti-HCV assays incorporating additional nonstructural proteins are being used as screening tests[24]. These kits for anti-HCV detection target different HCV antigens and detect more than five primary antibodies to ensure the specificity and sensitivity of the detection kit.

Anti-C22c and anti-C33c may be the first HCV antibodies to appear during the acute phase of the disease, which is defined by elevated alanine aminotransferase (ALT) levels and/or clinical symptoms[25]. Anti-NS5 appears somewhat later, while anti-C100-3 is the last antibody to be detected in acute self-limited HCV infection. The diagnosis and differentiation of acute from chronic HCV infection poses another problem. Patients chronically infected with one HCV-genotype develop acute hepatitis on infection with another genotype. Multiple episodes of acute hepatitis were observed in polytransfused thalassemic children reinfected with different HCV genotypes[26,27]. Therefore, discrimination between acute and chronic infection in the same patient is sometimes very difficult. HCV RNA in the serum or liver appears to be the earliest detectable marker of acute HCV infection, preceding the appearance of anti-HCV by several weeks[25]. HCV viremia may persist despite the normalization of serum ALT levels. Thus, the use of ALT levels in the diagnosis of HCV is not helpful. However, HCV RNA in serum usually lasts for fewer than 4 mo in patients with acute self-limited HCV infection. The average time from transfusion to sero-conversion is approximately 11 to 12 wk with EIA-1 (Enzyme immunoassay-1) and 7 to 8 wk with EIA-2 (Enzyme immunoassay-2). Now attempts are being made to develop EIA assays to differentiate HCV sub-types[28]. Patients with post-transfusion chronic non-A, non-B hepatitis develop anti-HCV antibodies in the majority of cases. Anti-HCV antibodies are not neutralizing, especially with HCV envelope proteins E1 and E2[29]. High levels of anti-C100-3 were correlated with high titers of circulating HCV in chimpanzees[30]. Therefore, the development and persistence of diagnostic antibodies to HCV seem to reflect concomitant virus replication and consequently a high

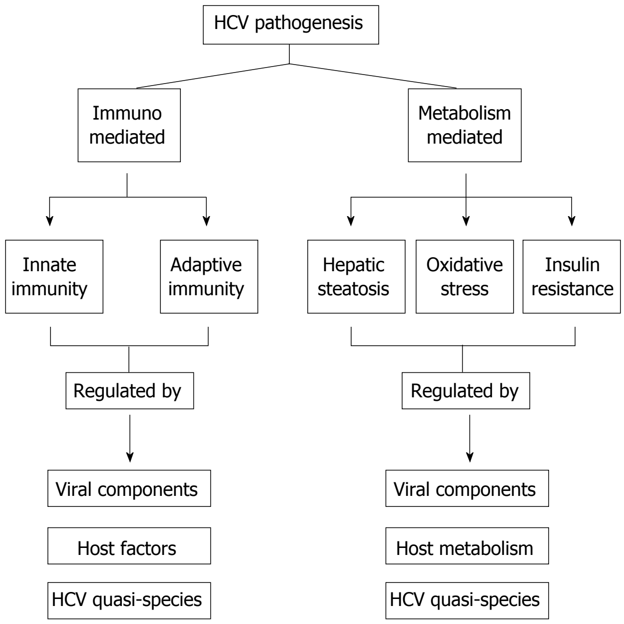

Figure 2 Regulation of hepatitis C virus pathogenesis by host immunity and metabolic factors. HCV: Hepatitis C virus.

potential for infectivity.

HCV RNA is frequently detected in patients with chronic hepatitis C and in patients carrying anti-HCV antibodies. A study carried out in Hong Kong demonstrated that 83% of anti-HCV positive patients were viremic when HCV RNA was determined using polymerase chain reaction (PCR) with two different sets of primers for noncoding regions[27]. Similarly, in another study, 98 of 100 patients with chronic non-A, non-B liver disease were positive for antibodies by EIA-2, but all 100 patients were positive for HCV RNA by PCR. With the currently available EIA systems, chronic HCV infection can readily be identified in most patients. Measurement of HCV RNA by PCR does not substantially increase the numbers of patients found to have chronic HCV infection[31]. Following the introduction and wider use of real-time PCR, it is now easier to diagnose and monitor the progress of HCV viremia in a very short time period[32]. In addition, the use of multiplex PCR by real time is another advancement in the detection of possible hepatitis viral co-infections in single attempt analysis[33].

Based on published information regarding various aspects of HCV infection including the currently available diagnostic assays and therapeutic regimens, the American Association for the Study of Liver Diseases and Centers for Disease Control and Prevention, United States have approved a document as "practice guidelines" for use in the diagnosis and treatment of HCV infection. This is an important document and describes details of the guidelines to be followed for laboratory diagnosis of acute/chronic HCV infection[34].

PATHOGENESIS OF HCV INFECTION

HCV is a non-cytopathic virus[35] that enters the liver cell and undergoes replication simultaneously causing cell necrosis by several mechanisms including immune-mediated cytolysis in addition to various other phenomena such as hepatic steatosis, oxidative stress and insulin resistance. The proteins/peptides encoded by different sub-genomic regions of the HCV genome and their quasispecies influence the above mechanism, and thus, have a significant role in HCV pathogenesis and disease causation. A brief description of HCV pathogenesis in the light of these factors is given in the following section (Figure 2).

Viral entry

HCV is a blood-transmitted virus that reaches the liver *via* circulation. The entry of HCV isolates requires at least 4 host-derived factors including scavenger receptor class B type I, Occludin, Claudin-I (CLDNI) and CD81. In addition, CLDN6 and CLDN9 have been shown to substitute for CLDN1 as HCV entry factors in human non-liver cells[36]. The CD81 molecule on host cell surfaces acts as a viral receptor, which binds with the viral particle and facilitates its entry in the liver cell[37,38]. CD81 is expressed on the surface of almost all nucleated cells and complexes with a variety of other cell-surface receptors such as CD19 and CD21 on B cells, and sends a costimulatory signal to the cells[39]. The viral envelop protein, E2, binds to the major extracellular loop of CD8[40]. HCV shows multi-site binding and can also bind to several other molecules such as the receptor for low-density lipoprotein, the dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and its liver counterpart[41,42]. E2 is the most variable viral protein, and therefore, its interactions with CD81 have been reported to be strain-specific[43]. It has two hyper variable regions, HVR-1 and HVR-2 which undergo frequent mutations, possibly due to virus-neutralizing antibodies and HCV-specific cytolytic T lymphocytes (CTLs). HCV also has a high mutation rate due to the lack of proofreading ability of its RNA-dependent RNA polymerase. Therefore, HCV exists in several distinct, but closely related virus species within an infected individual. These species are called HCV quasispecies.

HOST IMMUNITY

Innate immunity

Innate immunity presents a first line defense for the control of HCV infection as it does for several other viral infections. During HCV infection, cells produce Type 1 IFN which prepares and induces the cells to resist infection, check viral replication, promote adaptive immunity and activate natural killer (NK) cells, DCs and Kupffer cells *etc*. Once inside the cell, the innate immunity *vs* HCV is triggered through host recognition of viral macromolecular motifs, known as pathogen-associated molecular patterns (PAMPs), as non-self by cellular pathogen recognition receptors. These receptors includes toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs)[44]. RIG-I binds PAMP on

HCV-RNA and activates interferon regulatory factor-3 (IRF-3) for expression of IFN-α/β and anti-viral/interferon stimulated genes (ISGs)[45]. The secreted IFN and cytokines then activate NKs, DCs and Kupffer cells *etc.* These cells also play a significant role in mounting T/B cell-based immunity[46]. The PAMP region lies on the 3' untranslated region (UTR) of HCV and induces RIG-1 signaling[47] that results in a RIG-1 interaction with IFN-β promoter stimulator (IPS-1) which causes activation of IRF-3 and nuclear factor κB (NFκB).

HCV can effectively evade innate immunity resulting in persistent viral infection. This occurs because HCV has evolved to counteract the RIG-1 pathway[48] and thus evade the immune challenge. This phenomenon is the reason for chronicity in the majority of HCV infected patients. For this, the non-structural proteins of HCV *i.e.,* NS3 and NS4A form a complex which activates the NS protease domain to target cleavage of IPS-1. After cleavage, IPS-1 can no longer signal downstream to activate IRF-3 and NFκB and the infected cells no longer produce IFN-β or express ISGs[49].

NK cells, a major arm of innate immunity, play an important role in eradication of HCV. The liver is enriched in NK cells that are usually activated in an early phase of HCV infection. The activated NK cells recruit virus-specific T cells and induce antiviral immunity in the liver. They also eliminate virus-infected hepatocytes directly by cytolytic mechanisms and indirectly by secreting cytokines including IFN-γ and TNF-α. These cytokines induce an antiviral state in host cells. Surprisingly, HCV has evolved multiple strategies to counter the host's NK cell response. It is interesting that activated NK cells contribute toward liver injury, while inactive or compromised NK cells permit the virus to continue invasion[50].

Adaptive immunity

After entry and replication of the virus inside liver cells, the viral molecules are transported to the endoplasmic reticulum and associate with major histocompatibility complex (MHC) molecules, which are finally transported to the cell surface. These molecules on the cell surface are recognized by T cells for their immune action. The majority of CTLs are $CD8^+$ and recognize antigens presented on MHC class I molecules. Approximately 10% of CTLs are $CD4^+$ which recognize antigens presented on MHC II molecules. These CTLs eliminate cells infected with virus. However, HCV is reported to have evolved mechanisms to avoid recognition by CTLs. They either reduce the expression of MHC molecules or prevent the viral peptide from presentation at the cell surface. Thus, CTLs play a major role in viral eradication[51] and immunopathogenesis of HCV infection[52].

In another pathway of the disease mechanism, the destruction of HCV-infected hepatocytes release HCV fragments that are taken up by myeloid DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on HLA class II molecules. Subsequently, they increase expression of costimulatory molecules (CD80, CD86) which interact with and activate antigen-specific helper T (Th) cells[53]. These activated Th cells promote the maturation of DCs and increase the expression of CD40 ligand and TNF-α. The mature DCs induce T-cell activation by overexpression of their surface molecules. They also enhance antigen presentation capacity *via* HLA-I and production of cytokines that stimulate T-cell activation. IL-12 has been shown to play an important role in stimulating IFN-γ production from activated T cells[54,55], and thus, induces development of the type 1 (Th1) immune response characteristic of CTL activation. The effector CTLs release perforin, granzyme, and TNF-α, or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes[56,57].

The hepatocytes infected with HCV and DCs produce Type I IFNs which suppress viral replication by inducing enzymes such as 2'-5' oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes[58]. The plasmacytoid DC recognizes HCV-related markers through TLR-7, which interacts with single-stranded RNA[59]. The TLR-signaling up-regulates PDC-triggering receptor expressed on myeloid cells (PDC-TREM) which induce further production of IFN-α[60]. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysomes of viral mRNA[58]. When HCV-specific CTL responses are not strong enough to eradicate the virus this leads to persistent infection[61].

Successful clearance of HCV during acute HCV infection depends on the rise, vigor and persistence of the Th1 immune response[62,63]. Patients who developed a strong Th1 response showed efficient viral clearance and a self-limited disease course. In contrast, those who lacked IL-12 and IFN-γ production invariably developed chronic persistence of the virus. The majority of patients fail to control the infection and develop a chronic infection with a variable degree of hepatitis and viremia[64,65]. Experimental studies have also demonstrated that HCV components induce an antigen processing mechanism and IFN-stimulated genes in infected livers[66-68]. Impaired function of DCs, as antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. Various studies have reported that viral proteins including HCV core, E1, and NS3 inhibit DC maturation[69,70]. HCV infects DCs through the binding of HCV E2 protein and thereby suppress DC function in promoting an antiviral effect[41,71].

CTLs activated by viral proteins, not only kill virus-infected cells, but also contribute to virus control through a noncytolytic mechanism by secreting cytokines, *e.g.*, IFN-γ, IFN-α/β and TNF-α. These cytokines induce an antiviral state in host cells. This also renders uninfected cells resistant to infection and prevents viral replication. The progression of the majority of infected persons to chronic infection suggests inability of the antiviral immunity to contain this infection. There may be several reasons for this failure, including the emergence of escape variants as a result of a high rate of virus mutations, decreased production of antiviral cytokines or "stunning"

of HCV-specific CTLs, a compromised cytolytic potential of the CTLs and antagonistic peptides[72].

It is important to note here that the HCV genome in a single host is a dynamic population of different, but closely related genomes, designated quasispecies. The generation of quasispecies is usually ascribed to high variation in hyper variable region-1 (HVR-1) during viral replication[73]. In acute resolving hepatitis, HVR-1 shows very little variation, as compared to that in chronic hepatitis[74]. HVR-1 induces anti-HCV neutralizing antibodies[75,76] and HVR-1 specific $CD4^+$ and $CD8^+$ T cells[77,78]. Using the responding host cellular immune response differentially, HVR-1 favors viral escape[79,80]. HVR-1 variations result from the action of a continuous immune-driven positive selection[81,82]. Thus, HVR-1 complexity helps in the virus adaptive strategy to escape the immune onset. HCV clearance is associated with a vigorous HCV specific $CD4^+$ and $CD8^+$ T cell response in the acute phase of infection. In contrast, viral persistence is associated with a weak and dysfunctional virus specific T cell response[79-83]. T cell failure and HCV immune evasion have been explained in several reports[84-86].

Role of T regulatory cells in adaptive immunity

Recent studies have suggested a possible role for different regulatory T cell populations in HCV persistence. These studies showed a higher frequency of $CD4^+CD25^+$ regulatory T cells in the blood and $CD4^+FoxP3^+$ T cells in the liver of chronically HCV infected patients[87-89]. $CD4^+CD25^+$ regulatory T cells suppress HCV specific $CD8^+$ T cell and $CD4^+$ T cell proliferation as well as $CD8^+$ T cell IFN-γ secretion[87,90-92]. After HCV antigen stimulation, Treg cells secrete IL-10 and transforming growth factor-β (TGF-β) which suppress virus specific T cell responses[91-93]. $CD4^+CD25^+$ Treg cells obtained from chronically HCV infected patients demonstrated greater suppressive activity against HCV specific $CD8^+$ T cells compared to Treg cells isolated from acute HCV infected patients. However, the suppressive effect observed in patients who successfully cleared the virus was still significant[90]. Another study showed that the frequency of $CD4^+CD25^+FoxP3^+$ Treg cells and their suppressive capacity against virus specific T cell responses were as high in HCV recovered chimpanzees as those in persistently HCV infected chimpanzees[94]. This observation requires further in-depth studies to explore the actual suppressive effect of Treg cells during HCV infection. Induction of Treg cells by HCV antigens was first demonstrated by the response of $CD4^+$ T cell to HCV core protein. HCV-specific IL-10 secreting T cells were detected in the blood of chronic HCV infected persons[95]. Regulatory CD8+ T cells may play an important role in chronic HCV infection. HCV-specific $CD8^+CD25^+FoxP3^+$ T cells from the blood of chronically infected patients suppress HCV-specific T cell responses *via* TGF-β secretion. The blockade of TGF-β markedly enhanced HCV specific IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells[96].

Few other studies have shown that chronic HCV infection results in exhaustion or impairment of HCV-specific $CD8^+$ T cells. During chronic HCV infection, $CD8^+$ T cells fail to proliferate or secrete antiviral cytokines including IFN-γ. This phenomenon is promoted by a lack of $CD4^+$ T cells and the expression of immunomodulatory cytokines such as IL-10[97]. The major cause of HCV-specific $CD8^+$ T cell impairment is ascribed to the expression of inhibitory receptors such as Programmed death-1, lymphocyte-activation gene-3 (a protein related to CD4), CTLA-4 (a member of the CD28 receptor family), T-cell immunoglobulin mucin-3 and 2B4 on HCV-specific $CD8^+$ T cells in blood and liver[98]. Expression of these inhibitory receptors is associated with low levels of CD127 expression and impaired proliferation and differentiation of T cells. Thus, different mechanisms contribute to the dysfunction of HCV-specific $CD8^+$ T cells in chronic HCV infection.

In addition to cytotoxic T lymphocytes, humoral immune response against viral and cellular components during HCV infection is also present. Patients positive for HCV RNA and/or anti-HCV antibodies have type I anti-liver kidney microsome antibodies, which also recognize cytochrome P450 (CYP) 2D6. The patient's liver is infiltrated with auto reactive mononuclear cells, which recognize CYP2D6. It is interesting that the viral core protein residues 178-187 bear sequence homology with human cytochrome P450 (CYP2A6 and CYP2A7) residues 8-17[96]. Although HCV is a hepatotropic virus and infects hepatocytes, viral genome and its replicative intermediates are frequently present in peripheral blood mononuclear cells and lymphoid tissues of chronically infected persons. The viral glycoprotein E2 has been implicated in the oligoclonal expansion of several lymphoma cells[99]. The most common rheumatic and cutaneomucous symptoms in HCV-infected patients include fatigue, arthralgia, paraestheisa, myalgia, pruritus, and the sicca syndrome[100].

ROLE OF VIRAL PROTEINS AND GENOTYPES

The role of structural and non-structural components of the HCV virion has been explained by variation in their interactions with metabolites affecting pathogenic pathways leading to liver damage. HCV-core protein has a prominent role in all these interactions as compared to envelope and non-structural proteins. Moreover, when the mechanism of this interaction was studied in relation to various HCV genotypes, it was observed that different genotypes behave differently to regulate all these pathogenic pathways.

The role of NS5A and E2 region was found be important. NS5A has a role in viral replication, inactivating PKR[101-104], blocking the apoptotic pathway, binding of growth factor receptor-bound protein 2[105,106] and induction of anti-inflammatory interleukin secretion[107,108]. Similarly, E2 protein inhibits PKR[109,110]. The region of NS5A which interacts with PKR, shows clustering of amino acid changes during IFN treatment and plays an

important role in the evasion mechanism[111]. Furthermore, this association varies with genotype and thus, alters their sensitivity to IFN treatment. NS5A remains under strong immune selection, has T- and B-cell epitopes and possibly, in combination with individuals' HLA, selects immune cells to produce sensitivity/resistance to IFN therapy[112]. The functional activity of NS5A towards immune selection is clearly governed by the HCV-genotypes and varies accordingly. The response of genotype 2 and 3 to IFN treatment may be due to individuals recognizing the NS5A protein immunologically[13].

Binding of HCV E2 protein to DCs induces their maturation. Several HCV viral proteins, including core, NS3, NS5A and NS5B proteins, have been shown to inhibit DC functions[69]. Consequently, the functions of both $CD4^+$ Th cells and $CD8^+$ CTLs are impaired in chronic HCV patients. This has been suggested to be one of the mechanisms that HCV utilizes to weaken host immune responses and spread the infection. Indeed, many clinical studies have shown that in chronic HCV patients, not only the functions of DCs are impaired[113,114], the functions of both $CD4^+$ and $CD8^+$ T cells are also impaired[115]. A similar inductive effect of E2 protein was also reported in other cell types, including T cells, B cells[116], hepatocytes[117] and hepatic stellate cells[118].

The role of HCV genotypes in the progression of liver disease is one of the most controversial areas of HCV research. In patients with chronic HCV, infection with genotype-1b is reportedly associated with a more severe liver disease and a more aggressive course than the infection with other HCV genotypes. Similarly, it was found that HCV genotype-1b was significantly more prevalent among patients with liver cirrhosis and those with decompensated liver disease requiring liver transplantation than among those with chronic active hepatitis C[119-121]. Although this is indirect evidence, it suggests an association between HCV genotype-1b and the development of these complications. HCV genotype-1b is a marker for more severe HCV associated liver disease, because it reflects a longer time of infection than a mere aggressive form of hepatitis C.

METABOLIC CONDITIONS AFFECTING HCV PATHOGENESIS

In addition to immune mediated HCV pathogenesis, there are several other clinical and metabolic conditions that have a strong association with HCV pathogenesis. These include HCV-induced insulin resistance, oxidative stress and hepatic steatosis. The following is a brief description of the conditions affecting HCV pathogens:

HCV-induced insulin resistance

HCV infection influences overall metabolism leading to increased steatosis, fibrosis, inflammation, apoptosis and insulin resistance (IR)[122,123] during the course of the disease. The resulting IR shows a modulating impact on liver pathogenesis by HCV infection[124]. IR increases the *de novo* lipogenesis *i.e.*, fatty acid (FA) synthesis *via* overexpression and maturation of SREBP-1c. This in turn, increases the activities of lipogenic enzymes including Acetyl CoA carboxylase and FA synthase. At the same time, intermediates of triglyceride biosynthesis also activate inhibitors of insulin signaling. For example, activation of protein kinase C-E by phosphorylating insulin receptor substrate, and thus inhibiting phosphatidyl inositol-3,4,5-triphosphate[125], inhibits Akt translocation by ceramides *etc.*[126]. HCV-core protein, either directly or *via* increased secretion of TNF-α, causes IR[127,128]. The HCV core can activate inhibitors of insulin signaling including mammalian target of rapamycin[129] and suppressor of cytokine signaling (SOCS)-3 and C-Jun N-terminal kinase (JNK)[130,131]. The activation of JNK by HCV core may follow a direct or indirect proinflammatory cytokine-mediated mechanism.

HCV-associated oxidative stress

Oxidative stress is reported to be an important part of HCV-induced liver damage. Previous studies investigated the role of different molecular components of HCV structure in modulating oxidative stress during HCV infection. HCV-core protein present within the outer membrane of mitochondria induces oxidation of glutathione and promotes Ca^{2+} uptake into mitochondria. Clément *et al*[96] explained the molecular mechanism and demonstrated that following glutathione oxidation, there is increased reactive oxygen species (ROS) production by mitochondrial electron transport complex Ⅰ and Ⅲ. The HCV non-structural protein, NS5A, promotes ROS production in the membrane of endoplasmic reticulum (ER) by activating the release of Ca^{2+} from ER, thereby inducing oxidative stress[97]. NS3 protein induces ROS production by activation of NADPH oxidase[97]. Increased ROS production and consequent oxidative stress is evident by the presence of markers of increased oxidative stress in the blood. Levels of 8-hydroxy deoxyguanosine and 4-hydroxy-2-nonenol are increased in HCV infection[132,133]. Similarly, few studies have shown reduced levels of glutathione during HCV infection. Another study showed that the serum level of thioredoxin, a marker of oxidative stress, was significantly reduced in HCV infection[134-136].

The presence of oxidative stress has been noted in different types of hepatitis including hepatitis B. However, there is a marked increase in oxidative stress (OS) in HCV infection[132]. Several studies have shown that structural components of HCV induce effective OS[132]. HCV-core and non-structural components, NS3 and NS5A proteins, directly induce OS[137-139]. Core protein is involved in OS generation *via* oxidation of mitochondrial glutathione and uptake of Ca^{2+} into mitochondria[139,140] thus, changing the permeability of its membrane[141]. Electron transport complex Ⅰ increases production of ROS and redistributes cytochrome from mitochondria to the cytosolic fraction[93]. NS5A is associated with the ER membrane[142] and activates signal transducer transcription and NFκB[107]. These activations lead to inflammation,

immune response and apoptosis[143]. Similarly, NS3 triggers ROS by activating NADPH oxidase 2 in mononuclear and polymorphonuclear phagocytes[144] which increase apoptosis of hepatocytes[144]. All these reports conclude that the structural and non-structural components of HCV induce a significant increase in OS that results in liver damage during HCV infection.

HCV-induced steatosis

HCV infection is reported to have a strong association with hepatic steatosis. There are several other factors also responsible for steatosis, which include alcohol consumption, obesity, and diabetes[145-147]. Studies on steatosis in relation to hepatotropic viruses demonstrated that HCV infection directly causes steatosis in some patients[148]. Studies in experimental animals have shown that HCV-core protein promotes liver steatosis[149,150]. Furthermore, when steatosis was studied in relation to HCV-genotypes, it was noted that although steatosis is induced by all HCV-genotypes, it appears more prominent and frequent with HCV-genotype 3 infection[151-153]. In patients carrying genotype-3 infection, there was a good correlation between the level of steatosis and HCV replication[153,154] and the presence of HCV-core in the liver. In addition, steatosis resolves in patient with genotype-3 when treated successfully with anti-viral therapy as compared to those with non-genotype-3 who remain steatotic[155,156]. Steatosis reappears with relapse of infection[155]. This clearly demonstrates that some HCV-genotypes have more steatogenic potential. Subsequent studies[157] indicated that genotype-3 interferes with very low-density lipoprotein (VLDL) secretion. Core protein, which promotes lipid accumulation in hepatocytes[158,159], was more efficient from genotype-3 compared to core protein from genotype-1.

All these reports concluded that HCV causes steatosis in three different ways: (1) Impaired secretion of lipids from hepatocytes; (2) Increased *de novo* synthesis of free fatty acids (FFAs); and (3) Impaired FA degradation. The first aspect of HCV-induced steatosis was proposed due to the impaired secretion of VLDL. To substantiate this, reports from different studies demonstrated a decreased level of apolipoprotein B and cholesterol in chronic HCV infected patients[159,160]. These low levels pointed to HCV disturbing the assembly and secretion of VLDL from the liver[161]. Another important aspect in this relationship was increased *de novo* synthesis of FFAs in the presence of HCV infection. In this context, it is suggested that HCV upregulated the sterol regulatory element binding protein-1c (SREBP-1c) signaling pathway[158] with NS2 and NS4B proteins inducing SREBP at the transcriptional level[162,163]. SREBP was also induced by expression of HCV core protein. Studies in chimpanzees infected with HCV also demonstrated that HCV increased the activity of lipogenic enzymes such as ATP citrate lyase[164]. HCV-core, in particular, activates and helps in cellular lipid synthesis[164], possibly *via* its binding with retinoid receptor.

HCV-induced steatosis is also due to impaired FA degradation by HCV. Expression of HCV-core protein is reported to reduce the expression of peroxisome proliferation activated receptor-α (PPARα), a nuclear receptor involved in FA degradation and down-regulation of mitochondria β-oxidation[165]. Genotype-3 shows significant down-regulation of PPARα as compared to genotype-1[166,167]. HCV-core protein also down-regulates PPARα and therefore, is more effective when from genotype-3 as compared to genotype-1. The core protein from genotype-3 also down-regulated the PPARγ and up-regulated SOCS-7 in human hepatoma cells[167]. These data clearly show that HCV-core protein may modulate the expression of various genes responsible for FA degradation *via* down-regulation of PPARs.

CONCLUSION

HCV infection, previously known as blood borne non-A, non-B infection, is a serious public health problem worldwide. The diagnosis of HCV is based on the detection of anti-HCV antibodies and/or viral nucleic acid in serum. Studies over the last few years have developed assays not only for the accurate serodiagnosis of infection, but also identification of HCV serotypes. The pathogenesis of HCV infection is quite complex and regulated by host immunity as well as several metabolic activities influencing liver function. Whereas both innate and adaptive immunity are involved in the pathogenic action of HCV, the cytotoxic lymphocytes are crucial in deciding the eradication or persistence of viral particles. Moreover, the persistence of HCV infection is also affected by viral proteins, HCV isotypes and liver metabolism. In order to understand HCV pathogenesis further investigations are needed.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Scotomas in molecular virology and epidemiology of hepatitis C virus

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Abstract

In the 1970s, scientists learned of a new pathogen causing non-A, non-B hepatitis. Classical approaches were used to isolate and characterize this new pathogen, but it could be transmitted experimentally only to chimpanzees and progress was slow until the pathogen was identified as hepatitis C virus (HCV) in 1989. Since then, research and treatment of HCV have expanded with the development of modern biological medicine: HCV genome organization and polyprotein processing were delineated in 1993; the first three-dimensional structure of HCV nonstructural protein (NS3 serine protease) was revealed in 1996; an infectious clone of HCV complementary DNA was first constructed in 1997; interferon and ribavirin combination therapy was established in 1998 and the therapeutic strategy gradually optimized; the HCV replicon system was produced in 1999; functional HCV pseudotyped viral particles were described in 2003; and recombinant infectious HCV in tissue culture was produced successfully in 2005. Recently, tremendous advances in HCV receptor discovery, understanding the HCV lifecycle, decryption of the HCV genome and proteins, as well as new anti-HCV compounds have been reported. Because HCV is difficult to isolate and culture, researchers have had to avail themselves to the best of modern biomedical technology; some of the major achievements in HCV research have not only advanced the understanding of HCV but also promoted knowledge of virology and cellular physiology. In this review, we summarize the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

Key words: Hepatitis C virus; Hepatitis C virus lifecycle; Molecular virology; Hepatitis C virus models; Epidemiology

Core tip: The review summarizes the advancements, as well as remaining scotomas, in the molecular virology of hepatitis C virus (HCV). We emphasize the contributions of HuH-7 hepatocellular carcinoma cell line to development of the HCV replicon, cell culture-derived HCV, and HCV pseudoparticles. In addition, we reiterate the importance of epidemiological issues because accurate assessment of HCV-related disease burden has been overlooked. This review provides a history of the fight against HCV, which has required scientists to avail themselves to the best of modern biomedical technology, which in turn has enriched our knowledge of virology and cellular physiology.

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INTRODUCTION

The hepatitis C virus (HCV) is an enveloped, single-

stranded, positive-sense RNA virus, classified as a *Hepacivirus* within the *Flaviviridae* family[1-3]. The 9.6-kb RNA genome contains one long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR)[4-6]. The single ORF encodes an approximately 3000 amino acid (aa) polyprotein that undergoes co- and post-translational cleavage by host and viral proteases to yield 10 viral proteins, not including the F protein[7,8]. The structural proteins, nucleic acid-binding nucleocapsid core protein and envelope proteins (E1 and E2/P7) are encoded by 25% of the N-terminal portion of the genome[9]. The remaining 75% of the genome encodes the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B[9].

Humans are the primary reservoir of HCV[10]. HCV transmission occurs primarily through exposure to infected blood and the majority of individuals with persistent infection develop chronic hepatitis, which can progress to cirrhosis or hepatocellular carcinoma[11-13]. Different from other viruses, such as influenza A viruses and human immunodeficiency viruses, HCV is difficult to isolate and culture[14-16]. Since HCV was identified in 1989[1], basic research on HCV has been being hindered by the absence of reliable, reproducible, and efficient culture systems[11]. Recently, tremendous advances in understanding the HCV replicon[15,16], the pseudo-typed HCV viral particle[17], cell based culture systems[18,19], receptors[20-24], life cycle[25,26], structural biology and HCV therapy strategy[27-29] have been gained. However, several scotomas in the molecular virology and epidemiology of HCV remain to be elucidated. This review summarizes the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

MAJOR PROGRESS IN FIGHTING HCV

Since HCV was identified in 1989[1], virological research has led to a great deal of progress in the pathogenesis, diagnosis, treatment, control and prevention of the disease[11,30]. Since virus elimination is the ultimate goal of viral disease therapy, here we emphasize two major recent achievements in hepatitis C treatment. The first achievement was the development of direct-acting antiviral (DAA) agents, which are inhibitors of the HCV protease[31-37]. Although peginterferon and ribavirin remain vital components of therapy, the emergence of DAA agents has led to an unprecedented improvement in sustained virologic response rates to approximately 94%[30,38]. This is indicative of two milestones in virology: a therapy with the highest documented antiviral effects and optimism that the virus could be eliminated by medications. The second achievement is the identification of several single-nucleotide polymorphisms associated with spontaneous and treatment-induced clearance of HCV infection[39,40]. This discovery is also a milestone because only the rare single nucleotide polymorphisms of rs12980275 and rs8099917, near the interleukin28B gene, have any reported biological effect[39,40].

CURRENT HCV MODELS

The *in vitro* and *in vivo* models for HCV have evolved significantly since the discovery of the virus. With any virus, cell-based culture systems and animal models are the essential tools for virological study, vaccine development, and antiviral drug discovery. Many viruses, such as the influenza virus, are easy to isolate and culture in cell lines[41,42]; however, HCV is difficult to isolate and propagate[15,16,18,19]. Before HCV was identified, many virologists had attempted to isolate and culture the pathogen of non-A, non-B hepatitis using traditional cell-based approaches[43]. After struggling for decades, it was determined that HCV could only survive in human or chimpanzee fetal liver cells and hepatocytes or human peripheral blood mononuclear cells[44-49]. These cells are inconvenient to obtain and have a finite lifespan in culture, so even though these early studies showed that HCV was selective with a narrow host range[50,51], these methods made little contribution to HCV research (Table 1).

Defeated by classical approaches to isolate HCV, virologists were forced to reproduce the HCV lifecycle using split models, which included the HCV genome RNA replication model (HCV replicon)[15,16], HCV structural proteins model (virus-like particle, VLP)[52] and HCV pseudotyped viral particles model[17] (Table 1). In 1999, Bartenschlager's group in Germany established a HCV replicon system[15], followed soon after in 2000 by Rice's group in the United States[16]. These models simulated the structure of the subgenomic selectable HCV replicons composed of the HCV 5'-UTR, the gene encoding the neomycin phosphotransferase or firefly luciferase, the encephalomyocarditis virus internal ribosome entry site, the region encoding HCV NS2-5B or NS3-5B, the authentic 3'-UTR, and the 12-16 5'-terminal codons of the core[15,16]. The replicon could replicate autonomously in hepatic cell cultures (*e.g.*, HuH7 cell line)[15,16], leading to a series of experiments that examined the function of 5'- and 3'-UTR and NS3 to NS5B in HCV genome replication, described the viral life cycle, and led to the development of antiviral drugs[27]. The HCV replicon was able to replicate itself within the cell; however, it was not capable of producing infectious viruses[27]. Furthermore, this replicon was not able to reproduce in HuH-7 cells with high efficiency and for an extended period of time[50]. Virologists attempted to improve the replication efficiency and modify the robust HCV replicon using several methods, including adaptive mutation hunting, to reduce the non-HCV genome and increase the HCV genome composition, by attempting to replace various wild HCV strains of different genotypes[27,50,53].

Pseudotyped viral particles are commonly known as lentiviral vectors. These vectors are composed primarily of three viral elements; the gag-pol, which forms the viral structure, recognizes the viral genome and is responsible for the genome lifecycle; the viral mimic genome, which provides the genome elements that will be recognized

Table 1 Summary of *in vitro* and *in vivo* models for hepatitis C virus

In vitro and *in vivo* models	Established year	Advantages	Deficiencies
In vitro			
Cultivation of HCV	1993-1999	Achieved cultivation of HCV in human foetal liver cells, human hepatocytes or PBMC. Illustrated HCV is quite species selective and has a narrow range of hosts	Requires specific cellular factors to support viral lifecycle. Primary human and chimpanzee hepatocytes or highly differentiated cells dependent. Most of them have yielded limited success. Poor reproducibility and low levels of HCV replication
HCV replicon	1995-2000	Provided a cell-based model for the study on HCV genome replication	
HCV VLP	1998-1999	Rare evidence to support that HCV structural proteins core, E1, and E2 could form VLP	
HCVpp	2003	Provided a convenient and feasible tool for studies on viral entry, HCV receptor, neutralizing antibody, *etc.*	
HCVcc	2005	A break through in production of infectious hepatitis C virus in tissue culture	
In vivo			
Chimpanzee	1979	The only recognized animal model for HCV study, played a critical role in HCV discovery and play an essential role in defining the natural history of HCV	Chimpanzees differ from humans in their course of infection, that chronic carriers do not develop cirrhosis or fibrosis, limited availability, cost performance, and public resistance
Tree shrew	1998	Might be a succedaneum for chimpanzees	Persistent HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia. Germ line was not available to a small animal model
Chimeric human liver mouse	2001	Exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum. HCV can be transmitted horizontally. Drug evaluation	Since the mice were immunodeficient, they were not appropriate models to study HCV pathogenesis
Genetically humanized mouse	2011	Represents the first immunocompetent mice model for HCV study. Allows for the studies of HCV coreceptor biology *in vivo*	Operation is difficult

HCVpp: Hepatitis C virus (HCV) pseudotyped viral particles; VLP: Virus like particle; HCVcc: Cell culture derived HCV.

by gag-pol and ensures complete viral RNA metabolism; and the envelope proteins, which are presented onto the artificial viral particle[54-58]. Additionally, a lentiviral vector contains a reporter gene inserted into the artificial viral genome. Although the lentiviral vector was used widely in gene transduction, presenting an HCV envelope protein functionally in this viral particle was not considered. Virologists tried to generate the HCV VLP[52], because classic virological experience told us that VLP of a certain virus could be produced by cloning and expressing virus structural proteins, and Liang's group at the National Institutes of Health (Bethesda, MD) was successful in establishing the HCV-like particles using a baculovirus expression vector system[52]. In 2003, French virologist Bartosch *et al*[17] produced HCV pseudoparticles (HCVpp) using viral elements derived from murine leukemia virus. The HCVpp system led to advanced studies that identified a neutralizing antibody against HCV[59], explored HCV receptors and described the structure and function of the HCV envelope proteins[60-63].

In 2005, the Japanese virologist Wakita obtained a genotype 2a HCV strain (JFH-1) from a Japanese patient with a rare case of fulminant hepatitis C[18]. Based on the experience and methods accumulated in studying the HCV replicon, Wakita and his group rescued HCV in the HuH7 cell line, which was designated as HCVcc, for cell-culture-derived HCV[18]. HuH-7 cells infected with cloned and *in vitro* transcribed JFH-1 genomes produced viruses that were capable of infecting naïve HuH-7 cells[18]. In addition, the virus particles could be neutralized with a monoclonal antibody against the viral glycoprotein E2[18]. The study was the first *in vitro* experiment that showed the complete lifecycle of HCV. More importantly, virus obtained from the cell cultures was highly infectious in chimpanzees and immunodeficient mice with partial human livers[64].

As early as the 1970s, it was known that the etiological agent responsible for non-A and non-B hepatitis could be transmitted to chimpanzees[65], and chimpanzees were subsequently recognized as the only animal model of HCV[66] (Table 1). Chimpanzees played a critical role in defining the natural history of HCV[66] and since they are closely related to humans, any study of chimpanzees could reflect more closely what happens in humans than other animal models. However, chimpanzees that are chronic carriers of HCV do not develop cirrhosis or fibrosis[66,67], which are the most important consequences of HCV infection in humans.

Because chimpanzee studies are expensive and restricted by ethical responsibilities[67], scientists diverted their attention to other small animal models, such as the tree shrew and a chimeric human liver mouse. Xie *et al*[68] demonstrated that *Tupaia* could be infected by HCV when severely immunosuppressed; however, persistent

HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia[51]. By genetically manipulating the urokinase-type plasminogen activator transgenic mouse, Mercer *et al*[69] transplanted normal human hepatocytes into severe combined immunodeficient mice carrying a plasminogen activator transgene. The chimeric mice exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum[69]. Since the mice were immunodeficient, they were not appropriate models for investigation of HCV pathogenesis, although they were useful in assessing the activity of antiviral drugs, as well as neutralizing antibodies[51] (Table 1).

Mouse models of HCV provided little information about the human hepatocellular factors required for HCV entry. Thus, Ploss *et al*[24] introduced human CD81, scavenger receptor type B class 1, claudin 1, and *OCLN* genes into mice using a recombinant adenovirus expression system. They found that mice expressing these human factors were sufficient for HCV infection[24]. This system allowed for the investigation of HCV co-receptor biology *in vivo* and evaluation of passive immunization strategies and, therefore, represented the first immunocompetent small animal model for HCV[70] (Table 1).

SCOTOMAS IN MOLECULAR VIROLOGY

Although much progress has been made in all aspects of HCV research in the last few decades, we are still far from achieving the ultimate goal of complete HCV control and prevention. Thus, a better understanding of the HCV life cycle is essential to optimize the antiviral strategy. As mentioned above, the major challenges to HCV research are that HCV is difficult to isolate and culture, and no vaccine is available[14-16,71]. However, rather than summarizing the many achievements in HCV research, we have chosen to enumerate the scotomas in HCV molecular virology.

Structural biology of the HCV particle

Since HCV was first proposed to be a distinct infectious pathogen, virologists of that era attempted to visualize this enigmatic microbe using electron microscopy[43]. Different from other hepatitis viruses, including hepatitis A virus, hepatitis B virus and hepatitis E virus and the other viruses within the *Flaviviridae* family[43], no clear electron microscope image of HCV was reported until Chisari's and Rice's groups provided high-resolution images of highly enriched cell culture-derived HCV (HCVcc) particles in 2010 and 2013, respectively[72,73]. The reason for the difficulty in observing the crude HCV particle in HCV-harboring tissue remains unclear. The viral titer should not be an issue since viral copies in blood samples produced by HCV RNA are > 10^6/mL[74]. Serum-derived HCV particles are associated with the lipoprotein components apolipoprotein A-I (apoA-I), apoB-48, apoB-100, apoC-I and apoE[75]. The interaction between virus particles and serum lipoproteins suggests that HCV may form hybrid lipoviral particles[75] that facilitate virus entry into hepatocytes and protect the virus from the host immune response. In addition, the lipoprotein components might affect the morphological observation of crude HCV particles by electron microscopy. Our current knowledge of HCV morphology indicates that HCV particles are 40-80 nm in diameter, pleiomorphic, lack obvious symmetry or surface features and contain electron-dense cores[72,73]. The lack of details describing the overall architecture of HCV limits the ability of molecular biologists to study HCV structural biology and topology.

Molecular virology of HCV structural proteins

A quarter of the N-terminal region of the HCV polyprotein encodes the core structural protein and glycoproteins E1 and E2, which are believed to be incorporated into the HCV particle[17]. Based on general virological knowledge, the core protein should be a major component of the viral capsid, responsible for viral genome RNA recognition, binding and packaging[27]. Glycoproteins E1 and E2 located in the viral surface are also called envelope proteins; E1 and E2 are responsible for receptor recognition, receptor binding, endocytosis and membrane fusion[27]. The mature core protein contains a positively charged N-terminal RNA binding domain and a C-terminal domain that consists of two amphipathic helices and a palmitoylated cysteine residue to facilitate peripheral membrane binding[27]. Antibodies against core proteins are important for HCV serological detection[43]. Previous studies demonstrated that the core protein is involved in many pathogenic processes[43]. Furthermore, the core protein induces hepatocellular carcinoma in transgenic mice[76] and is a potent inhibitor of RNA silencing-based antiviral response[77]. However, the basic function of the core protein in capsid formation remains unknown. Although the region between amino acids 82 and 102 contains a tryptophan-rich sequence involved in homotypic core proteins interaction[78], the HCV core particle had not been successfully produced. *In vitro* nucleocapsid reconstitution experiments using the 1-124 or 1-179 core segments and structured RNA molecules have yielded irregular particles larger than those reported by the limited electron microscopy observations[79]. Scientists in China at Xiamen University successfully generated human papillomavirus VLP[80], hepatitis E virus VLP[81] and hepatitis B virus core capsids but failed to produce the HCV capsid (personal communication). In addition, the crystal structure of the HCV core protein is not yet available (Table 2).

E1 and E2 are type Ⅰ transmembrane glycoproteins assumed to be class Ⅱ fusion proteins, with N-terminal ectodomains of 160 and 334 amino acids, respectively, and a short C-terminal transmembrane domain of approximately 30 amino acids[27,43]. Studies of HCVpp have indicated that 14 amino acids from the HCV core and 12 amino acids from the E1 C-terminus are required for E1 and E2 function[62]. The hemagglutinin and neuraminidase of influenza A viruses matches each other in a

Table 2 Summary of the properties of hepatitis C virus structural proteins

	Core	E1	E2	p7
Genome location	342-914	915-1490	1491-2579	2580-2769
Translation processing site	Rough ER			
Amino acid composition	191	192	363	63
Molecular weight (kDa)	21-23	33-35	70-72	7
Glycosylation	No	Yes	Yes	No
Cleavage	ER signal peptidase and SPP			
Crystal structure	Not available			Revealed
Functional unit	Dimer	Heterodimer?		Hexamer
Common function	Viral particle formation. Core, E1 and E2, together with p7 and NS2, are required for virus assembly (assembly module)			
Unique function	Capsid protein, viral particle formation, viral genome recognizing and packaging. Interacts with cLDs in early viral particle formation process. Counters host antiviral factors and involves pathogenesis	Envelope glycoproteins, interact with SRB1, CD81, CLDN1, OCLN, *etc.* to trigger viral entry. Promote fusion with the endosomal membrane. Counter host immune response *via* hypervariable regions		Viroporin. Has key roles in organizing the virus assembly complex. p7-NS2 complex interacts with the NS3-4A enzyme to retrieve core protein from cLDs to form viral particle
Major scotomas	How do the core form the viral capsid? The signals and processes that mediate RNA packaging are largely unknown. What impeded us to resolve the structure of the viral glycoproteins? What is the real process in HCV entry? How are these receptors and co-receptors temporally and spatially used to ensure the early infection processes?			

Start co-ordinates based on H77 (accession number, NC_004102). SRB1: Scavenger receptor class B member 1; CD81: Tetraspanin CD81; CLDN1: The tight junction protein claudin 1; OCLN: The tight junction protein occludin; cLDs: Cytosolic lipid droplets; ER: Endoplasmic reticulum; SPP: Signal peptidase and signal peptide peptidase; HCV: Hepatitis C virus. The molecular weights of E1 and E2 refer to the glycosylated forms.

relative slack manner regardless of gene homology[54-58], while the matching pattern of HCV E1 and E2 is relatively strict. We separated E1 and E2 of HCV genotypes 1a, 1b, and 2a into two individual expression plasmids and replaced the transmembrane domains of 1b and 2a E1 and E2 with that of genotype 1a. The complementation features of E1 and E2, as well as the contributions of both the ecto- and transmembrane domains to the formation of the E1E2 complex, were evaluated using the HCVpp system[63]. We found that 1aE2 could not only complement its native 1aE1 but also 1bE1; in genotype 1b, glycoprotein complex formation is dependent primarily on the overall biological characteristics of the intact native E1 and E2; in genotype 2a, although the interaction of intact native E1 and E2 is critical for the formation of the glycoprotein complex, the ectodomain made a greater contribution than did the transmembrane domain[63]. This study suggested that E1 and E2 formed a functional envelope protein complex dependent on E1 and E2 expression[63]. E1 and E2 are assembled as non-covalent heterodimers[82,83], although the number of E1 molecules necessary to aggregate with E2 for biological function has not been elucidated. We highlight this scotoma because the envelope proteins of many viruses do not function simply in a 1:1 ratio[54-58] and viral proteins are not translated in equal numbers[43]. A lack of understanding of this point will impede receptor discovery, regardless of how many receptors and co-receptors are identified[75]. The crystal structure of the dengue virus glycoprotein, which is another member of the *Flaviviridae* family, was revealed in 2004[84]. By contrast, virologists failed to produce the HCV E1 or E2 crystal, which limits our understanding of the biological characteristics of HCV envelope proteins. Since one of the most important biological functions of HCV envelope proteins is membrane fusion, E1 and E2 are assumed to be class Ⅱ fusion proteins[83]. This assumption has been challenged by recent studies suggesting that HCV and pestiviruses share an uncharacterized mechanism of membrane fusion[85]. These contradictory issues are common in HCV research and await further advances in our understanding of HCV virology (Table 2).

Molecular virology of non-structural HCV proteins

HCV proteins can be categorized into an assembly module (from core to NS2) and a replication module (from NS3 to NS5B) on the basis of viral essential functions[27,75]. Details on the function of structural and non-structural HCV proteins are lacking due to the limitations of *in vitro* models. There is also some controversy on topics such as whether the assembly module is necessary for viral particle formation or whether p7 is a structural or non-structural protein. Although the HCV replicon system provided solid evidence that non-structural proteins activate HCV RNA replication *in vitro*[15,16], some unresolved issues remain. These include why it is not possible to turn this system into a fully competent HCV cell culture model or why all replicons, except for the genotype 2a JFH-1 clone, contain cell-culture-adaptive mutations that when introduced back into viral genome, render it non-infectious in chimpanzees[67].

The p7 polypeptide is a small, 63-aa intrinsic membrane protein with a double-membrane-spanning topology in which its N- and C-terminal ends face the ER lumen[27]. Recent data indicate that p7 can mediate membrane ion permeability and form hexamers[86,87]. The three-dimensional structure of a hexameric p7 channel revealed a highly tilted, flower-shaped protein architecture with six protruding petals oriented toward the ER lumen[86,87]. These structural and membrane-permeability

Table 3 Summary of the properties of hepatitis C virus non-structural proteins

	NS2	NS3	NS4A	NS4B	NS5A	NS5B
Genome location	2769-3419	3420-5312	5313-5474	5475-6257	6258-7601	7602-9378
Translation processing site	Rough ER					
Amino acid composition	810-1026	1027-1657	1658-1711	1712-1972	1973-2420	2421-3012
Molecular weight (kDa)	21-23	70	8	27	56-58	65-68
Cleavage	Viral cysteine protease NS2-3 and the serine-type protease activity of the viral NS3-NS4A complex					
Crystal structure	C-terminal (aa904-1026) was solved	Revealed	Revealed	Not available	Revealed	Revealed
Functional unit	Homodimer	Monomer or oligomer	Monomer	Oligomer	Homodimer	Monomer
Common function	Replication module					
Unique function	A metal-dependent proteinase, many functions dependent on the interaction with P7 and NS3. Participation in proteolytic cleavage at the NS2-NS3 junction of the polyprotein. Both the TMDs and protease domain of NS2 are required for the production of virus particles	The DAA targeting protein. NS3 was anchored in ER membrane by cofactor NS4A. NS3-4A complex has serine-type protease activity and NTPase/RNA helicase activities. Nonspecific cleavage of two critical interferon induction proteins: MAVS and TRIF	The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication as well as assembly	A master organizer of replication complex formation. NTPase activity? RNA binding?	Produced as multiple phospho-variants. RNA-binding phosphoprotein involved in RNA replication. Phosphorylation of a specific serine residue within the C-terminus by CK Ⅱ α is essential for virus assembly. The interaction of NS5A with the cLD-bound core protein is the key steps in HCV assembly	RNA-dependent RNA polymerase
Major scotomas	How HCV particles are organized? What is the accurate duty of each nonstructural protein in viral lifecycle? How do the nonstructural proteins utilize host cellular factors for its own survival? Why HCV lifecycle is tightly associated with components of LDLs and VLDLs?					

Start co-ordinates based on H77 (accession number, NC_004102). Aa: Amino acid; TMD: Transmembrane domain; CK Ⅱ: Casein kinase Ⅱ; cLD: Cytoplasmic lipid droplet; LDL: Low-density lipoprotein; VLDL: Very-low-density lipoprotein; MAVS: Mitochondrial antiviral signaling protein; TRIF: TIR-domain-containing adaptor inducing interferon; HCV: Hepatitis C virus.

properties suggest that p7 belongs to the viroporin family and could play an important role in viral particle release and maturation[86,87]. However, the role of p7 in calcium and ion metabolism is unknown. Furthermore, HCVpp with or without p7 showed no changes in viral particle formation and pp infectivity[62,63]. A study of the closely related GB virus B, which infects tamarins and has an analogous but larger protein, p13, showed that p13 is processed into two components p6 and p7, and that p6 was dispensable while p7 was essential for infectivity[88].

NS2 is a metal-dependent proteinase, whose functions are dependent on the interaction with p7 and NS3[27]. Although the NS2 protease is dispensable for RNA replication, NS2 participates in proteolytic cleavage at the NS2-NS3 junction of the polyprotein[27]. The transmembrane and protease domains of NS2 are required for infectious virus assembly[89]. Why NS2 is critical for viral particle formation remains unknown and the interactions between NS2 and other structural and non-structural viral proteins to form an unknown viral particle formation network should be explored (Table 3).

NS3 is a 70-kDa multifunctional protein anchored by the cofactor NS4A[27,89]. NS2/NS3 junction cleavage is essential to liberate fully functional NS3 protein[27,89]. NS3-4A is a non-covalent complex, with a serine protease located in the N-terminus (aa 1-180) and an NTPase/RNA helicase in the C-terminus (aa 181-631)[27,89]. The substrate specificity of NS3-4A is low and causes non-specific cleavage of host proteins; *e.g.*, mitochondrial antiviral-signaling (MAVS) and TIR domain-containing adaptor inducing interferon β (TRIF), and thus might impact host IFN response[90]. The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site[27]. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication, as well as assembly[27]. The DAAs telaprevir and boceprevir[31-37] are inhibitors targeting the NS3-4A protease that displayed promising effects in clinical trials, indicating that the NS3-4A protease is critical for viral life cycle (Table 3).

NS4B is a poorly characterized hydrophobic 27-kDa protein[27] comprised of a 66-aa N-terminal portion, a 120-aa central portion, and a 70-aa C-terminal portion[91]. Four transmembrane-spanning regions were predicted in the central portion, while the N-terminal portion plays an important role in assembly of a functional replication com-

plex[27,91]. Einav *et al*[92,93] and Thompson *et al*[94] demonstrated that NS4B harbors NTPase activity and has a role in viral assembly.

NS5A is a 447-aa membrane-associated protein that plays an important role in modulating HCV RNA replication and particle formation[91]. NS5A can be detected in basally phosphorylated and hyper-phosphorylated forms with molecular weights of 56- and 58-kDa, respectively[95,96]. NS5A is comprised of four domains: a N-terminal membrane anchor and three domains separated by two low complexity sequences[27,91]. The three separated domains are domain 1, aa 36-213; domain 2, aa 250-342 and domain 3, aa 356-447. Domains 1 and 2 are involved in RNA replication and domain 1 is involved in cellular lipid drop binding, domain 3 is essential for viral assembly and is involved in interaction with the core protein accumulated in cellular lipid drops[27,91]. Although studies showed that NS5A is critical for HCV RNA replication, deletions in D2 and D3 are tolerated in RNA replication[97], and viable replicons and viruses harboring GFP insertions displayed no change on HCV RNA replication[98]. Phosphorylation of a specific serine residue within C-terminal by casein kinase Ⅱα is essential for virus assembly[99]. The interaction of NS5A with the cytosolic lipid droplets-bound core protein is a key step in HCV assembly[97,100,101].

NS5B is an RNA-dependent RNA polymerase (RdRp). Its crystal structure was revealed in 1999[102]; the active site is highly conserved and located in the palm subdomain[91]. The low substrate specificity allows for the incorporation of ribavirin into nascent RNA. Thus, ribavirin remains a perfect RNA analog in HCV therapy[103]. Although recombinant NS5B is available and its crystal structure is known[104,105], its role in HCV RNA replication remains unclear (Table 3).

5’-non-translated regions and 3’ non-translated regions

Viral non-translated regions (NTRs) and non-coding regions, harbor important biological functions, involving viral genome reorganization, replication, translation initiation, and viral assembly[106]. The HCV 5’NTR contains 341 bp (H77 strain, NCBI Reference Sequence: NC_004102.1)[107]. The predicted secondary structure of the HCV 5’NTR consists of four domains (domains Ⅰ-Ⅳ, numbered from 5’ to 3’), and the largest domain Ⅲ was further categorized into sub-domains a-f[107]. The major functional unit in the HCV 5’NTR is an IRES, which includes three domains (Ⅱ-Ⅳ)[106-109]. Initiation of protein synthesis in host cells utilized by HCV is different from mRNA translation in eukaryotes because HCV initiates viral protein synthesis *via* its IRES, which is known as internal translation initiation. This process is a cap-independent mechanism of recruiting, positioning and activating the host cellular protein synthesis machinery driven by the HCV IRES[106-109], which is relatively weak in directing protein translation compared to the IRESs of other viruses, and may contribute to an insufficient host immune response[110]. Although structural and biochemical studies of the IRES found in HCV have provided the most detailed information thus far regarding the mechanism of IRES driven translation, unresolved issues remain. For example, it is unknown whether the HCV IRES acts as one determining factor for hepatotropism or how the HCV 5’NTR interacts with the 3’NTR to support HCV RNA replication and polyprotein translation. Additionally, the biological impact of the NTR to each hepatitis virus remains unclear since the HCV NTRs have a different structure compared to those of hepatitis A and E viruses. Finally, the interaction of miR-122 with the HCV 5’NTR to facilitate replication of viral RNA remains to be fully elucidated[111].

The 3’ terminal of any genome is technically difficult to identify and the available complete sequence of the HCV 3’NTR is unusual. The 3’ UTR is divided into three structurally distinct domains from 5’ to 3’, an upstream variable region of about 40 nucleotides, a long poly (U)-poly (U/UC) tract and a 98-nucleotide (3’ X) sequence that forms three stem-loop structures[43,106]. The long poly (U)-poly (U/UC) tract was a major obstacle to obtaining an HCV genomic clone because no known DNA polymerase could amplify this region and the fidelity of a reverse transcriptase in this region was suspect. The function of the 3’ UTR remains to be determined[43,106]. It may play an important role in minus intermediate RNA and genome RNA synthesis during HCV RNA replication[43,106] since variable region deletions of RNA replicons could replicate, albeit at a much lower level[112]. However, deletion of either the poly (U/UC) or the 3’ X was not viable, which suggested that the poly (U/UC) and 3’X regions are critical for HCV RNA replication[107].

HuH7 cell line

HCV researchers should be familiar with the human hepatocellular carcinoma cell line HuH-7, also known as Huh7 or HuH7. This cell line is critical because the HCV replicon, HCVcc, and HCVpp are all dependent on this cell line or its derivatives, indicating that it harbors all critical factors for HCV replication, assembly, budding and entry[43,113-115]. HuH-7 is a well-differentiated hepatocyte-derived hepatocellular carcinoma cell line that originated from a liver tumor in a 57-year-old Japanese male in 1982 (http://huh7.com/). It was established by scientists at Okayama University of Japan in the 1980s (http://cellbank.nibio.go.jp/legacy/celldata/jcrb0403.htm). HuH-7 remains the only hepatocellular carcinoma cell line that can fully support the HCV life cycle. Improvements in hepatocellular carcinoma cell line isolation could provide more effective HCV-supporting cell lines; alternatively the advances in induced pluripotent stem cells could result in a breakthrough in HCV culture and isolation.

SCOTOMAS IN EPIDEMIOLOGY OF HCV

HCV carries a large disease burden in some countries and is the second most studied virus. Most HCV infections are subclinical with a long and insidious disease

Table 4 Epidemiological features of hepatitis C virus infection

Epidemiological index	Current consensus
Source of infection	Chronic HCV carriers
Route of transmission	HCV transmission occurs primarily through exposure to infected blood. Past: Receiving infected blood or organ transplantation, from accidental exposure to infected blood, and sexual transmission in persons with high risk behaviours. Present: HCV is usually spread by sharing infected needles with a chronic HCV carrier, and some people acquire the infection through nonparenteral means that have not been fully defined.
Susceptible population	General population
Incubation period	Average 6-10 wk
Prevalence and incidence	3% of the world's population have HCV
Rate of chronic infection	Up to more than 80%
Outcome of chronic infection	10%-20% of chronic HCV carriers may develop into cirrhosis and liver failure. 1%-5% of chronic HCV carriers are associated with the development of hepatocellular carcinoma
Molecular epidemiology	HCV is classified into eleven major genotypes (designated as 1-11), many subtypes (designated a, b, c, *etc*.), and about 100 different strains (numbered 1, 2, 3, *etc*.) based on the genomic sequence heterogeneity. Genotypes 1-3 have a worldwide distribution. Types 1a and 1b are the most common, accounting for about 60% of global infections. Type 2 is less frequently represented than type 1. Type 3 is endemic in southeast Asia and is variably distributed in different countries. Genotype 4 is principally found in the Middle East, Egypt, and central Africa. Type 5 is almost exclusively found in South Africa, and genotypes 6-11 are distributed in Asia.
Stability	HCV is inactivated by exposure to lipid solvents or detergents, heating at 60 ℃ for 10 h or 100 ℃ for 2 min in aqueous solution, formaldehyde (1:2000) at 37 ℃ for 72 h, β-propriolactone and UV irradiation.
Vaccine	Not available

HCV: Hepatitis C virus.

course. However, epidemiological surveillance of HCV is relatively weak compared to some acute respiratory transmission diseases (http://www.who.int/influenza/surveillance_monitoring/en/).

Chronic HCV carriers are the only reservoir of HCV since chimpanzees could be infected with HCV only experimentally[43,106]. HCV transmission occurs primarily through exposure to HCV-infected blood[43,106]. Blood transfusion, solid organ transplantation from an infected donor, and unsafe medical practices were the major transmission routes before HCV was identified in 1989[43,106]. Beginning in the early 1990s, strict screening of blood donors and precise control over the blood supply were implemented by national governments[43,106,116]. The majority of HCV infections are now limited to specific subpopulations, such as intravenous drug users and patients with certain hemopathies[117]. Although unsafe medical practices, occupational exposure to infected blood, maternal-fetal transmission, sex with an infected person and high-risk sexual practices are believed to be HCV transmission routes, the rate of acquisition of infection by these routes is low[118]. The average incubation period is 6-10 weeks and most virologists and hepatologists consider that up to 80% of HCV infected persons do not eliminate HCV spontaneously[43,106]. Cirrhosis and liver failure develop in 10%-20% of chronic HCV carriers; 1%-5% of chronic HCV carriers develop hepatocellular carcinoma[43,106] (Table 4).

The World Health Organization estimates that up to 3% of the global population is infected with HCV (http://www.who.int/csr/disease/hepatitis/Hepc.pdf), and the peak disease burden is expected around 2020[119]. However, these estimations lack sufficient evidence. Firstly, it is hard to track the origin of the data since most authors citing this statistic used inaccurate citations. Secondly, the HCV serological detection kit has undergone at least three iterations[117]. The first test developed in 1990 detected antibody to a single epitope within the core protein by enzyme linked immunosorbent assay and provided data on 170 million HCV carriers, even though it was plagued by poor sensitivity[43]. Third-generation enzyme immunoassays included antibodies against multiple antigens, which increased the sensitivity significantly[43], although no large-scale serological investigations have been performed. In China, a nationwide HCV serological survey performed in 2006 showed the prevalence of anti-HCV antibodies to be < 0.5% among more than 80000 Chinese subjects[116]. Furthermore, the rates of HCV were much lower than those of hepatitis B among clinical inpatient and outpatient populations, which was significantly different from a Japanese population[119,120]. Epidemiology is important because it will provide basic knowledge of disease and inaccurate epidemiological data will lead to inaccuracies in our knowledge of the disease burden, natural history and therapeutic efficacy.

The number of people that will become chronic carriers after HCV infection remains unknown. Scientists believe that as many as 40%-80% of HCV infections will develop into chronic infections[121-123] (Table 3). While these estimates are also likely inaccurate, how and when people are infected must be determined to ascertain a more precise figure. One recent cross-sectional study performed in intravenous drug users challenged the current assumptions regarding the rate of chronic infection; in that study as many as 77.8% of individuals cleared HCV infection without the need for anti-viral therapy[117].

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REVIEW

Liver function impairment in liver transplantation and after extended hepatectomy

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Abstract

Extended hepatectomy, or liver transplantation of reduced-size graft, can lead to a pattern of clinical manifestations, namely "post-hepatectomy liver failure" and "small-for-size syndrome" respectively, that can range from mild cholestasis to irreversible organ non-function and death of the patient. Many mechanisms are involved in their occurrence but in the recent past, high portal blood flow through a relatively small liver vascular bed has taken a central role. Therefore, several techniques of inflow modulation have been attempted in cases of portal hyperperfusion first in liver transplantation, such as portocaval shunt, mesocaval shunt, splenorenal shunt, splenectomy or ligation of the splenic artery. However, high portal flow is not the only factor responsible, and before major liver resections, preoperative assessment of the residual liver function is necessary. Techniques such as portal vein embolization or portal vein ligation can be adopted to increase the future liver volume, preventing post-hepatectomy liver failure. More recently, a new surgical procedure, that combines *in situ* splitting of the liver and portal vein ligation, has gradually come to light, inducing remarkable hypertrophy of the healthy liver in just a few days. Further studies are needed to confirm this hypothesis and overcome one of the biggest issues in the field of liver surgery.

Key words: Small-for-size syndrome; Liver transplantation; Extended hepatectomy; Liver failure; Cirrhosis

Core tip: In this review we focus on the small-for-size syndrome and post-hepatectomy liver failure, the most feared complications of liver surgery, fundamentally similar in pathogenesis and clinical manifestations, occurring when the residual liver is not large enough to accommodate the markedly increased portal vein blood flow. Our aim is to simplify a concept, which has been a major concern in hepatic surgery for some time. Many efforts have been and are being made to overcome such an important problem in this field.

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INTRODUCTION

The liver is a unique organ, capable of regeneration and functional recovery after parenchymal injury. When the volume is too small to satisfy the metabolic demand, the liver loses this peculiar ability, resulting in delayed synthetic dysfunction with poor bile production, coagulopathy, prolonged cholestasis and intractable ascites, which can lead to septic complications and high mortality. The

term "small-for-size syndrome" (SFSS) was first[1] coined in liver transplantation as a consequence of size mismatch between graft and recipient, an event occurring especially in the setting of living donor liver transplantation (LDLT) or split liver transplantation[2], where the use of partial grafts has gained worldwide acceptance to overcome the shortage of cadaveric organs. However, the same concept can also be applied to the field of liver resection, where patients with marginally resectable tumors are at high risk of developing post-hepatectomy liver failure (PHLF)[3], a clinical manifestation comparable to the SFSS.

DEFINITION

There is not full consensus about the definition of SFSS. It was introduced in 1996 by Emond *et al*[1] and regarded the clinical manifestation following transplantation of small grafts in LDLT. The term SFSS on the basis of personal working experience, and no threshold values of liver function tests, was suggested. In 2005, Dahm *et al*[4] proposed a more precise definition. These authors described SFSS after liver transplantation as the presence of two of the following criteria recorded on three consecutive postoperative days: serum bilirubin > 100 μmol/L (6 mg/dL), international normalized ratio (INR) > 2 and presence of encephalopathy grade Ⅲ or Ⅳ. The small-for-size syndrome usually occurs during the first postoperative week and is diagnosed after the exclusion of other causes such as technical complications (*e.g.*, arterial or portal occlusion, outflow congestion, bile leak) and/or rejection or infections (*e.g.*, cholangitis, sepsis).

The same concept is applicable to the field of hepatic surgery, where extended resections can lead to the development of PHLF. Many different definitions of PHLF have been proposed in the literature[5-7]. In trying to propose a more standardized definition, in 2011, Rahbari *et al*[7] suggested a simple and easily applicable definition of PHLF as a "postoperative acquired deterioration in the ability of the liver to maintain its synthetic, excretory and detoxifying functions, which are characterized by an increased INR and concomitant hyperbilirubinemia on or after postoperative day 5". They differentiated severity in three grades (A, B, C), according to whether changes in clinical management of the patient or invasive treatments are required. It is of interest that even if SFSS and PHLF can be viewed as the same manifestation of liver function impairment, the two terms and their relative definitions are currently separated. It would probably be of interest to join the two definitions into a single one, but at present no suggestions, regarding this topic, are present in the literature.

PATHOPHYSIOLOGY

The magnitude of the effect of increased portal flow after hepatectomy on the development of PHLF, though recognized, is currently not yet well established and most of the studies regarding this topic come from the transplantation experience.

High portal blood venous flow (PVF) has gained a central role in the pathogenesis of SFSS. Under normal physiological conditions, portal vein blood flow accounts for 75% of total hepatic inflow, or 90 mL/min per 100 g of liver tissue, while the hepatic artery contributes for 20%-25%[8]. The portal vein lacks intrinsic auto-regulation. Hence, after extended hepatectomy or transplantation of small grafts, the remnant liver is subjected to the portal flow destined to a whole liver, through a reduced micro-vascular bed[9]. Such a substantial increase of PVF and shear-stress on sinusoidal lining cells is inversely related to graft size. In > 75% partial hepatectomy, PVF increases by more than twice the baseline values, resulting in PHLF, with high morbidity and mortality[10]. Although shear-stress is considered to be a necessary stimulus for hepatic regeneration[11], excessive forces can be detrimental to both the function and survival of the reduced-size organ: the result is damage of sinusoidal spaces with release of inflammatory cytokines, responsible for progressive hepatocyte necrosis[12]. Pathological findings include hepatocyte ballooning, tremendous mitochondrial swelling, irregular large gaps between sinusoidal lining cells, and collapse of the space of Disse[13].

Although portal vein pressure (PVP) is considered a reliable predictor of graft failure[14], the latter and PVF do not run parallel to each other; furthermore, the lack of correlation between graft weight/recipient body weight ratio (GRWR) and PVP has been investigated[15].

Blood flow regulation, which allows a steady rate of hepatic perfusion, depends not only on the classical arterial intrinsic regulation but also on an inverse relationship between portal and hepatic arterial flow, also known as hepatic arterial buffer response (HABR)[16]. When the portal blood flow increases, this leads to an elevated wash-out of adenosine levels in the space of Mall, contracting the hepatic artery[17]. Adenosine is unlikely to be the sole vascular regulator and other vaso-active compounds may contribute to HABR[18]. The consequences of such a diminished arterial blood flow manifest in the peripheral circulation as a centrilobular microvesicular steatosis or infarcts, or, in severely affected cases, as ischemic cholangitis in the hilum[19]. Hence, the clinical manifestations can range from mild cholestasis to liver failure. However, the optimal rate needed to sustain liver regeneration and function, without damage to the liver, is still not known and further experimental studies on animal models are needed.

PREOPERATIVE PREDICTION

Hepatectomy remains the first curative option for neoplasms of the liver. The mortality rate after major liver resections, *i.e.*, the removal of three or more Coinaud segments, ranges from 3% to 7% in non-injured liver parenchyma and increases up to 32% in patients with cirrhosis[20]. Thus, the extent of parenchymal resection

Table 1 Predictive factors of small-for-size syndrome and post-hepatectomy liver failure

Liver volume	Liver function	Patient-related	Other
FLR/TLV	CHILD-PUGH	CALI	Cholestasis
GRWR or GV/SLV	HVPG	Age > 65 yr[37]	Liver stiffness[31]
	ICG	Male sex[37]	Donor factors[44]
	MEGX[30]	Diabetes mellitus[37]	

FLR: Future liver remnant; TLV: Total liver volume; GRWR: Graft weight-recipient body weight ratio; GV: Graft volume; SLV: Standard liver volume; HVPG: Hepatic vein pressure gradient; ICG: Indocyanine green clearance; MEGX: Monoethylglycinexylidide; CALI: Chemotherapy-induced liver injury.

is an essential parameter in establishing both the operability of each patient and the risk of PHLF and this, to date, is still a subject of debate, probably due to different methods of measurement, variability in the segment volumetric distribution and degree of underlying disease.

The 3D volumetric computed tomography reconstruction allows preoperative calculation of the liver volume, even of the single segments, and, more important, of the future liver remnant (FLR). With a normal function, FLR should range between 20% and 30% of total liver volume, whereas smaller volumes are correlated with increase of liver failure and infections[21,22]. Care must be taken when an underlying liver disease pre-exists. In "injured" livers, (steatosis, cholestasis, fibrosis, cirrhosis or chemotherapy) the FLR should be greater than 30%-40%[23]. Therefore, an accurate preoperative assessment of liver function is needed.

In patients with cirrhosis, the Child-Pugh score and the hepatic vein pressure gradient are the two most important restrictive criteria in selecting candidates for surgery[24,25] even if they do not provide precise assessment of liver resectability[26]. Metabolic tests based on the detoxifying properties of the liver have the advantage of providing a more reliable estimation of the hepatic function, and they are based on quantitative measures. Indocyanine green clearance is the most popular test[27], especially in Eastern countries, where it constitutes the pillar of preoperative algorithms for liver resection[28,29]. Other quantitative tests, such as the monoethylglycinexylidide[30] test, have led to good prediction of PHLF, but they have gained less popularity and are not routinely used. A simple and non-invasive method of measurement of liver stiffness (Fibroscan®) has recently been gaining broad consensus for predicting PHLF in selected patients[31], but further studies are needed to establish its potential role in patient selection for surgery.

Chemotherapy-induced liver injury is common in patients that received chemotherapy for colorectal liver metastases, and the two typical patterns are sinusoidal injury (sinusoidal obstruction syndrome) in oxaliplatin-based regimens, and steatohepatitis (CASH), associated with irinotecan treatment[32]. More than 6 cycles of oxaliplatin need a longer time interval before major hepatectomy, even though accountability for PHLF still remains a matter of debate[33], whereas irinotecan is associated with an increased risk of peri-operative mortality after hepatectomy[34]. Biopsy of the liver before surgery might be helpful to assess the grade of steatosis or the histological features of CASH, thus defining more precise windows between drug administration and surgery.

Cholestasis impairs liver regeneration, and levels of bilirubin above 2.9 mg/dL are related to a higher rate of liver failure after major hepatectomy[35]. Nevertheless, the use of preoperative biliary drainage is still controversial, except for acute cholangitis or small FLR that are candidates for portal vein embolization[36], in which case biliary drainage is highly recommended. Besides such patient-related factors, others, like age > 65 years, male sex and diabetes mellitus, are related to a high risk of PHLF[37]. Obesity is not per se a major predictor of liver failure[38].

In the setting of transplantation, liver volume assessment is represented by the GRWR or graft volume/standard liver volume ratio (GV/SLV): in LDLT safe thresholds are at least 0.8% of GRWR or 30%-40% of GV/SLV[2,39,40], with greater values in patients affected by portal hypertension or advanced chronic liver disease. There are reports on the successful use of smaller grafts[41], but in association with some intraoperative inflow modulations: a case report of a left lobe LDLT as low as 0.34% of GRWR underwent splenectomy and did not develop post-operative SFSS[42]. In liver transplantation, size is not always the sole factor responsible for graft post-transplant liver function[43], because graft quality is likewise important in order to avoid liver dysfunction or other complications. Aside from basic requirements for donor livers, the following donor factors have a negative impact on graft prognosis: age > 50 years, prolonged intensive care unit stay > 5 d, hypernatremia, prolonged cardiac/respiratory arrest and long ischemia times, administration of high dosage of vasopressors, severe systemic sepsis, steatosis > 30%, anatomic variations in vascular structure and, obviously, abnormal liver function, particularly with elevated serum bilirubin and gamma glutamyltransferase[44].

Prediction of SFSS and PHLF is feasible and is based on the calculation of liver volume up to the assessment of liver function. Evaluation of patient status can help to find the best candidate for surgery. In the field of liver transplantation, donor characteristics also have to be taken into account, defining which grafts are at higher risk of developing SFSS than others. A list of the above mentioned factors is shown in Table 1.

ATTENUATING SFSS IN LIVER TRANSPLANTATION

In the presence of high portal blood flow and/or small grafts (GRWR < 0.8%), several different technical flow manipulations can be performed to overcome graft hyperperfusion and reduce PVF, although there is no full consensus about their indications: portocaval shunt, mesocaval shunt, splenorenal shunt, splenectomy or ligation of the splenic artery. Boillot *et al*[45] reported the first

successful mesocaval shunt with downstream ligation of the superior mesenteric vein in a left lobe transplantation (GRWR of 0.61%), based on previous experimental studies on pigs.

Hemi-portocaval shunt, *i.e.*, anastomosis between the left or the right portal branch and the inferior vena cava in a permanent fashion, is advocated by Troisi *et al*[10] whenever the PVF at reperfusion exceeds three-four times the one recorded in the donor. None of the patients undergoing such a graft inflow modulation developed SFSS, with significant decrease of portal vein flow.

The effects of splenic flow diversion have been investigated in the presence of portal hypertension (PVP > 20 mmHg)[15] and/or of portal hyperperfusion (PVF > 250 mL/min per gram)[46]. However, when PVF exceeds 500 mL/min per gram, portosystemic shunt cannot be avoided.

Both splenic artery ligation (SAL) and splenectomy can be performed and are comparable in terms of outcome and overall survival[47], although for the latter, septic complications must always be taken into account. Splenectomy is considered superior to SAL for the purpose of increasing white balance and platelet count after LDLT, which is not achieved by SAL alone.

Splenic artery embolization represents a valid alternative to achieve portal decompression[48]. Furthermore, a linear correlation between PVF and graft-to-recipient spleen size ratio has been found, thus including the spleen size as a likely predictor of post-transplant portal hyperperfusion and SFSS[49].

Techniques of graft inflow modulation account for a certain risk of steal phenomenon[50]: portal vein thrombosis, encephalopathy, septic complications or hampered liver regeneration are described as principal side effects. It remains an open question whether and when portosystemic shunts should be removed[51], since hypoperfusion, as well as hyperperfusion, can also be detrimental for liver function.

According to the definition of Dahm *et al*[4], who stated that SFSS should be considered as a distinct entity, outflow obstruction per se should be excluded as a possible trigger, as it may reduce the hepatic function. However, one of the most discussed topics concerns the reconstruction of the middle hepatic vein (MHV) in right lobe grafts, since congestion of anterior segments (Ⅴ-Ⅷ) may lead to graft dysfunction[52]. A graft with inclusion of the MHV has been demonstrated to be technically and physiologically superior, but the use of this technique should be limited to selected cases in LDLT due to an increased risk for donor safety[53]. For MHV reconstruction, several transplant centers use various types of vascular grafts, with a predilection for large caliber autologous vessels (*i.e.*, the superficial femoral vein), or also cryopreserved venous or arterial grafts[54].

ATTENUATING PHLF IN EXTENDED HEPATECTOMY

If the remnant liver volume is not sufficient to meet the future metabolic demand, a number of strategies can be adopted to increase the liver volume, preventing post-hepatectomy liver failure. Portal vein embolization (PVE) has become the most standardized procedure due to its safety and feasibility: it consists in the occlusion of portal flow ipsilateral to the lesion, inducing hypertrophy in the contralateral lobe. Makuuchi *et al*[55] first used this technique in 1982 to extend the limits of hepatic resection, thus increasing the number of cases suitable for curative surgery: in this early report, 14 patients underwent pre-operative PVE followed by major liver resection 6-41 d after embolization, with no occurrence of postoperative liver failure. After almost 30 years, the indications of PVE are still very poorly standardized: many authors indicate a residual liver volume less than 30% of total liver volume or up to 40% in injured livers as the critical threshold[56,57]. Surgery is usually performed 2-8 wk after PVE, with future liver remnant volume increased by 10%-46%. From 70% to 100% of patients who underwent PVE, hemi-hepatectomy or extended hepatectomy could be performed. Following resection, the perioperative morbidity and mortality was less than 15% and 0%-7%, respectively[58-60].

Portal vein ligation (PVL) represents a good alternative, although there are no controlled studies clearly showing the superiority of PVE *vs* PVL. Portal vein ligation requires laparotomy and, furthermore, the volume gain is often limited due to formation of collaterals between the two different lobes[20]. PVL is not considered such a standardized and safe procedure as PVE, but patients who are candidates for 2-stage hepatectomy can benefit from this technique[61,62], recently adopted in a new surgical approach aimed at enhancing and accelerating the regeneration of the remnant liver[63]. In 2009, Schnitzbauer *et al*[63] reported on a case series of 25 marginally resectable patients with massive involvement of the right lobe by neoplastic nodules, on which an innovative 2-step technique was carried out. In the first step, right portal vein ligation and *in situ* splitting of the liver on the right side of the falciform ligament was performed; in the second step, after a median time interval of 9 d, extended hepatectomy (right trisectionectomy) was completed. The observed median increase in volume of the left lobe was 74%, but morbidity and mortality were significant (68% and 12%, respectively). Thereafter, the so-called advanced liver partition and PVL for staged hepatectomy, also known by the acronym ALPPS[64], has spread to many centers worldwide: the obtained median increase in volume ranges from 74% up to 87%, with surgery usually performed 5-30 d after the first step. However, mortality rates of 13%-22% are still reported[65-68]. Although the procedure is innovative and attractive, these latter figures make it imperative to increase the number of patients treated with this strategy to better define its feasibility and limits[69].

In addition to the above, more studies are needed to understand the exact mechanisms of hepatic regeneration, also through biopsy of the remnant liver before and after hepatectomy, and measurements of portal flow

and pressure should be provided. In fact, although the preserved functional capacity of the hypertrophied remnant liver could be established with functional tests (*e.g.*, indocyanine green clearance) and through the uptake of 99mTc dimethyl iminodiacetic acid[64], excessive portal flow represents one of the main problems, determining a possible discrepancy between the relevant increase in volume and the amount of actually functioning parenchyma. de Santibañes *et al*[70], in 2012, claimed that the diseased right hemi-liver, left in place, acts as an auxiliary liver to assist the future liver remnant for the first and critical week after resection, but in true auxiliary transplantation, both the portal and arterial flows to two hemi-livers are maintained. Thus, contrary to auxiliary transplantation, in which the growth and functional recovery may progress harmonically with a real portal flow modulation, this phenomenon is not certain after extended hepatectomy with a small residual parenchyma. In other words, how can this "beneficial" re-direction of the entire portal flow to a "small-for-size" remnant liver comply with established principles of portal flow modulation in small-for-size transplantation? Research in animal models clearly shows that a portocaval shunt has a positive effect in attenuating liver injury after extensive hepatectomy, suggesting that a slower regeneration following reduction of portal flow may be more advisable than faster regeneration associated with temporary portal hyperflow[71,72]. In this view, more insights on the mechanisms and features of liver regeneration are needed to better understand the potential benefit of portal flow modulation to prevent postoperative liver failure[64].

PHARMACOLOGICAL INTERVENTIONS

Many drugs have been demonstrated to be effective in attenuating SFSS after living donor liver transplantation of small grafts, but most of them have been tested only in animal models[73,74], whereas clinical trials on human beings are still lacking. Furthermore, pharmacological portal flow modulation has been investigated: shear-stress attenuation has been achieved by somatostatin[75], through down-regulation of the endothelin-1 (sinusoidal vasoconstrictor) and up-regulation of heme-oxygenase-1 (vasodilatator and antioxidant). Nitric oxide pathway activation seems to be protective against ischemia-reperfusion injury both in liver resection and liver transplantation[76]. Therapeutic agents promoting liver regeneration, such as serotonin, are still a matter of debate for their controversial role[77]. Recently, autologous bone marrow stem cells have been used to increase liver regeneration prior to major liver resection. In particular, an enhanced parenchymal growth after portal vein embolization through the portal injection of $CD133^+$ cells (in the non-embolized hepatic lobe) has been demonstrated, with a subsequent improvement of outcome after surgery[78]. Even though the specific effect of $CD133^+$ cells is not completely understood[79], this approach is intriguing due to the possibility of combination with other techniques favoring post-transplant or post-hepatectomy liver function recovery, such as procedures of portal flow modulation.

CONCLUSION

Post-hepatectomy liver failure and small-for-size liver syndrome can be viewed as two sides of the same coin, since both of them can lead to an identical pattern of clinical manifestations, that is cholestasis, impairment of coagulation and development of ascites, and that can range up to irreversible organ non-function and death of the patient. Safe thresholds of remnant liver volume differ between liver transplantation and after extended hepatectomy, probably due to graft denervation, immunosuppressive therapy and severity of ischemia-reperfusion injury. However, preoperative assessment of liver function and size is crucial, while intraoperative recording of hemodynamic changes, before and after hepatectomy or liver transplantation, should be mandatory in order to perform inflow modulation, if necessary. Other strategies, which include pharmacological perioperative protection of the liver and stem cell injection, are being explored, but further studies are needed before they can be applied in the clinical field.

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REVIEW

Exocrine pancreatic insufficiency in adults: A shared position statement of the Italian association for the study of the pancreas

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Abstract

This is a medical position statement developed by the Exocrine Pancreatic Insufficiency collaborative group which is a part of the Italian Association for the Study of the Pancreas (AISP). We covered the main diseases associated with exocrine pancreatic insufficiency (EPI) which are of common interest to internists/gastroenterologists, oncologists and surgeons, fully aware that EPI may also occur together with many other diseases, but less frequently. A preliminary manuscript based on an extended literature search (Medline/PubMed, Cochrane Library and Google Scholar) of published reports was prepared, and key recommendations were proposed. The evidence was discussed at a dedicated meeting in Bologna during the National Meeting of the Association in October 2012. Each of the proposed recommendations and algorithms was discussed and an initial consensus was reached. The final draft of the manuscript was then sent to the AISP Council for approval and/or modification. All concerned parties approved the final version of the manuscript in June 2013.

Key words: Exocrine pancreatic insufficiency; Chronic pancreatitis; Gastric surgery; Pancreatic surgery; Pancreatic neoplasms; Risk factors; Clinical studies

Core tip: Pancreatic exocrine insufficiency represents a condition related to pancreatic and extrapancreatic disease. We have reviewed the evidence related to the pathophysiological aspects of exocrine pancreatic diseases and we have also reported the recommendations for treating this condition in the most common pancreatic and extrapancreatic diseases. Pancreatin minimicrospheres is a drug which is cost-effective according to a survey of Polish patients, but studies demonstrating its cost-efficacy in Italy are necessary.

Pezzilli R, Andriulli A, Bassi C, Balzano G, Cantore M, Delle Fave G, Falconi M, Frulloni L; the Exocrine Pancreatic Insufficiency collaborative (EPIc) Group. Exocrine pancreatic insufficiency in adults: A shared position statement of the Italian association for the study of the pancreas. *World J Gastroenterol* 2013; 19(44): 7930-7946 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/7930.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.7930

INTRODUCTION

This is a medical position statement developed by the Exocrine Pancreatic Insufficiency collaborative group

which is a part of the Italian Association for the Study of the Pancreas (AISP). We covered the main diseases associated with exocrine pancreatic insufficiency (EPI) of common interest to internists/gastroenterologists, oncologists and surgeons, fully aware that EPI may occur in many other diseases, but less frequently (Giardia and HIV infections, lymphoma, Whipple's disease, amyloidosis).

LITERATURE SEARCH METHODS

A preliminary manuscript based on an extended literature search (Medline/PubMed, Cochrane Library and Google Scholar) of published reports was prepared, and key recommendations were proposed. A MESH term "EPI" was used for the search on Medline/PubMed and key words (exocrine pancreatic insufficiency) were used for both Cochrane Library and Google Scholar. A total of 1465 manuscript were retrieved on Medline/PubMed, 64 on Cochrane Library and 1234 on Google Scholar. After deduplication only 282 papers regarding the specific aims of the study were selected and 151 were utilized. The evidence and recommendations were discussed at a dedicated meeting in Bologna during the National Meeting of the Association in October 2012 in which was present 130 participants. Each of the proposed recommendations and algorithms was discussed and an initial consensus was reached. The final draft of the manuscript was then sent to the AISP Council for approval and/or modifications. All concerned parties approved the final version of the manuscript in June 2013.

PHYSIOLOGY OF PANCREATIC DIGESTION OF NUTRIENTS

The pancreatic secretion is a clear fluid liquid, 97% of which is water and electrolytes[1], and 3% proteins. In turn, these are made up of proteins (3%) mainly represented by proteases (80%), amylase (7%), lipase (4%) and nucleases (1%)[2]. The normal absorption of nutrients involves a complex mixture of digestive enzymes and bile salts, and an intact intestinal mucosa to enable the uptake of these hydrophobic complexes. Under normal condition, all major pancreatic enzymes act simultaneously with postprandial chyme decrease during duodenal-ileal transit[3]; the rate of intraluminal degradation differs widely among the major enzymes due to their different stability regarding inactivating mechanisms[4]. Pancreatic amylase is a very stable enzyme, probably because of its high resistance to enzymatic proteolysis[5]; the majority of that released into the duodenum reaches the terminal ileum in an active form[4,6,7] whereas approximately 60% of the protease activities released into the duodenum are delivered to the mid-jejunum, and only between 20% and 30% reach the terminal ileum[4]. As regards lipolytic enzymes, lipase is most susceptible to inactivation during small intestinal transit. In the absence of triglycerides, a large proportion of lipase activity is also lost between the duodenum and the jejunum, and only small quantities are delivered to the terminal ileum[4,5]. After ingestion, dietary lipids are initially emulsified in the stomach and then hydrolyzed by the action of gastric and pancreatic lipase and colipase; hydrolyzed lipids are then aggregated into micelles or liposomes with the addition of bile salts in the duodenum and jejunum, the micelles are absorbed across the intact intestinal villi by both active and passive processes and, finally, packaged into chylomicrons within intestinal epithelial cells and transported to the circulatory system via the lymphatic system[8].

Table 1 Causes of pancreatic insufficiency

Chronic pancreatitis
Primary pancreatic insufficiency
Agenesis of the pancreas
Congenital pancreatic hypoplasia
Shwachman–Diamond syndrome
Johanson–Blizzard syndrome
Adult pancreatic lipomatosis or atrophy
Isolated lipase or colipase deficiency
Pancreatic resection
Pancreatic cancers
Secondary pancreatic insufficiency
Mucosal small bowel disease: Decreased cholecystokinin release
Somastatinoma or exogenous somatostatin analog intake: Decreased pancreatic secretion
Gastrinoma: Intraluminal destruction of enzymes
Surgery and Billroth II anastomosis: Poor mixing or decreased hormone release, disturbance of innervations
Periampullary tumors (pancreatic duct obstruction)

Modified from reference 155.

MECHANISMS OF EXOCRINE PANCREATIC INSUFFICIENCY

Exocrine pancreatic insufficiency results from a progressive loss of acinar pancreatic cells which leads to the secretion of an insufficient amount of digestive enzymes into the duodenum. As indicated in Table 1, chronic pancreatitis is the most well-known cause of EPI[9] but also several other conditions, such as partial or total surgical resection of the gland, loss of function of pancreatic tissue or obstruction of the main pancreatic duct as well as diabetes, celiac disease, inflammatory bowel diseases, and gastrectomy should also be considered. Maldigestion results when exocrine (mainly lipase and trypsin) pancreatic function is reduced by more than 90%; other pancreatic and extra-pancreatic causes of maldigestion are reported in Table 2[10].

CLINICAL MANIFESTATION AND ASSESSMENT OF EXOCRINE PANCREATIC INSUFFICIENCY

Patient complaints

Patients with steatorrhea typically report an increase in daily bowel movements, with fatty, bulky stools which are

Table 2 Pathogenesis of maldigestion

Mechanism	Explanation
Decreased pancreatic production	Lack of functional tissue or decreased endogenous neurohormonal stimulation
Decrease in delivery	Pancreatic duct obstruction
Decreased activation	Low duodenal pH
Premature enzymatic degradation	Decreased contact time due to increased motility, impaired interaction with chyme and biliary salts, and intestinal bacterial overgrowth

difficult to flush away. This occurs mainly after high fat-containing meals and is sometimes not a daily symptom. As steatorrhea occurs after meals, it typically happens 2 to 3 times a day in individuals with a normal lipid-content diet. Weight loss and anorexia may also develop over time due to malnutrition.

Physical examination

Chronic malabsorption results in weight loss, such as temporal scalloping, interosseous wasting, and lack of subcutaneous fat. Nail leukonychia due to hypoalbuminemia may be present in the late stages of chronic malabsorption. Signs of liposoluble vitamin lack may appear; ecchymoses due to clotting abnormalities in the case of vitamin K deficiency, ataxia and peripheral neuropathy resembling Friedreich ataxia due to vitamin E deficiency, abnormalities of night blindness and xerophthalmia (dry corneas) due to vitamin A deficiency; contraction or muscle spasms, osteomalacia and osteoporosis may also occur due to hypocalcemia. Examination of the stool is an important tool for recognizing steatorrhea.

Investigations

Exocrine pancreatic function is currently diagnosed using two groups of tests, usually referred to as direct and indirect (or tubeless) tests; the principal tests are reported in Table 3. The most sensitive test is a direct test based on aspiration of the pancreatic contents during secretin or secretin-cholecystokinin/cerulein administration[11]; this test is only available in a few centers, it is invasive and is not indicated in clinical practice. Other tests currently available in clinical practice are indirect tests. At present, fecal elastase-1 determination is the most diffuse test for screening pancreatic exocrine insufficiency[12], usually using a monoclonal test[13]. This test does not require the withdrawal of enzyme supplementation therapy and is based on analysis of a single stool sample. Concentrations of elastase-1 less than 200 μg/g in feces are compatible with exocrine pancreatic insufficiency and less than 100 μg/g are indicative of severe pancreatic insufficiency[14]. The ^{14}C-triolein breath test and the cholesteryl-[1-^{13}C] octanoate breath test have been used for assessing fat malabsorption[15]; the *D*-xylose test (normal serum *D*-xylose concentration greater than 1.33 mmol/L 1 h after an oral dose of *D*-xylose) for exploring the malabsorption of carbohydrates, and fecal chymotrypsin excretion (normal > 6 U/g) for evaluating the malabsorption of proteins[12].

Two other tests, not presently available commercially, are the *N*-benzoyl-*L*-tyrosyl-p-aminobenzoic acid (PABA) test and the pancreolauryl test which are based on the recovery of an ingested dose of PABA and fluorescein dilaurate from the urine[16]. In clinical trials, objective confirmation of excess fecal fat may be undertaken, and the following methods are usually used[9]: Sudan staining of random homogenized stool, steatocrit and quantitative fat analysis. Sudan staining evaluates the number and size of fat globules per high-power field (hpf), and the test results are scored as normal (≤ 20/hpf, 1 to 4 micrometers in size), moderately increased (> 20/hpf, 1 to 8 μm in size) and definitely increased (> 20/hpf, 6 to 75 μm in size)[17]. Compared to chemical fat analysis, Sudan staining has a sensitivity of 94% and a specificity of 95% for diagnosing abnormal fecal fat excretion[18]. Steatocrit is a quantitative measurement of fat and is expressed as a proportion of an entire centrifuged homogenized stool sample[19]. A spot acid steatocrit level (normal < 10%) has been reported as having a sensitivity of 100% and a specificity of 95% when compared to 72-h quantitative fat analysis[20]. The best reported method is the 72-h fat chemical analysis using the van de Kamer method. The patients need to keep a food diary to ensure that adequate dietary fat (100 g/d) is consumed during the test; the normal output is less than 7 g of fat per 24-h period[21]. Coefficient of fat absorption (CFA) should be used to better quantify the steatorrhea; it is calculated using the following equation: CFA (%) = 100 [(mean fat intake - mean stool fat)/mean fat intake[22]; in healthy subjects, the CFA is usually greater than 80%[23].

A new assessment for pancreatic malabsorption which takes into consideration some serum parameters reflecting nutritional status (magnesium < 2.05 mg/dL, reduced serum levels of prealbumin, albumin, retinol binding protein, ferritin, and hemoglobin) has recently been reported[24], but it requires further validation[25].

Finally, bioelectrical impedance has been proposed for assessing nutritional status in patients with pancreatic cancer[26]. This method is based on the different conductive and resistive properties of the various body tissues; it is not invasive, it is inexpensive and it can be performed at the bedside. In brief, fixed low-voltage and high-frequency alternating current introduced into the body is conducted through the fluid compartment of the fat-free mass and it is able to measure both body resistance and capacitance. Capacitance causes the current to lag behind the voltage, creating a phase shift; this shift is quantified geometrically as the angular transformation of the capacitance: resistance ratio, also called phase angle.

Pancreatic enzyme replacement therapy

In order to avoid maldigestion and ameliorate the nutritional status of patients with EPI, the cornerstone of treatment is pancreatic enzyme replacement therapy (PERT). Available formulations contain pancreatic enzymes encapsulated in microgranules or minimicrospheres with a pH sensitive coating in order to either

Table 3 Indirect diagnostic tests for evaluating pancreatic exocrine insufficiency

Test	In favour	Against
CFA	Gold standard	72 h stool collection; 100 g standard diet; no simultaneous PERT; not pancreas specific
Acid steatocrit	Linear correlation with CFA also in a single sample; Good as screening	High fat diet needed; 24-72 h stool collection is ideal
Fecal elastase 1	Single stool sample; PERT can be continued	Poor sensitivity in mild EPI, watery stools and small bowel disease
13C-mixed triglyceride breath test	Simple; Also for mild forms of EPI and therapy assessment	Requires further validation
Fecal chymotrypsin	Good for compliance control; Single small stool sample	Sensitivity low for clinical practice (chymotrypsin is variably inactivated during intestinal transit); not for mild EPI; watery stools decrease enzyme activity; PERT must be discontinued
Secretin-enhanced magnetic resonance cholangiopancreatograpgy	Morphological and semi-quantitative functional changes	Requires further validation
Nutritional status (magnesium < 2.05 mg/dL, ↓prealbumin, ↓albumin, ↓retinol binding protein, ↓ferritin, ↓hemoglobin)	Simple	Requires further validation

CFA: Coefficient of fat absorption; PERT: Pancreatic enzyme supplementation therapy; EPI: Pancreatic exocrine insufficiency.

prevent the release and the subsequent inactivation of enzymes by gastric acidity or to release the enzymes into the intestinal lumen where the pH is higher and optimal for the digestion and absorption of food. Currently, the Italian guidelines also suggest minimicrospheres to be the ideal pancreatin formulation[9].

The initial recommended dose of pancreatic extract which should be given is 40000-50000 units of lipase per meal and 25000 U per snack, and this dose should be progressively increased until the steatorrhea is totally or sufficiently reduced[27,28]; this dosage should be maintained over time.

Dietary and drug recommendation

Food intake should be distributed between three main meals per day, and two or three snacks. The pancreatic extracts should be ingested during the meals.

Even if a diet which is low in fat reduces steatorrhea and improves maldigestion, it restricts caloric intake and is not a good option.

Medium-chain triglycerides (MCTs) have not been shown to be effective in patients suffering from chronic pancreatitis with EPI. Moreover, their poor palatability and high cost reduce patient compliance. Evidence exists that MCTs also require enzyme supplements for proper digestion and absorption[29]. They should be used only in patients with persistence of symptoms or weight loss despite adequate enzyme supplementation[30]. Medium-chain triglycerides have been proposed in PERT non-responders as an “ultima ratio”. The quantity of energy administered by MCTs is limited (ca 8.3 kcal/g) and the dose must be increased slowly in order to achieve intestinal adaptation, even when using enteral nutrition[31]. However, trials have shown no advantage between a normal balanced diet and MCT-enriched preparations[29,32,33].

A diet rich in fiber content is contraindicated because the fibrous material will interfere with proteolytic and amylolytic enzyme activity; lipolytic activity is most affected[30,34], whereas enzymes contained in gastroprotected minimicrospheres can be assumed also with food having a pH less than 5.5. Acid-suppressing agents should be utilized only in patients who continue to experience symptoms of maldigestion despite the adequate administration of PERT[35].

Goal of the treatment

Steatorrhea in severe pancreatic insufficiency is very difficult to resolve completely, and only a 60%-70% reduction is usually achieved using PERT[36]. This may be due the fact that there are numerous interactions between pancreatic maldigestion, intestinal ecology and intestinal inflammation; consequently, to the methods of achieving optimal management of pancreatic maldigestion need to be fully re-evaluated, considering not only the correction of pancreatic insufficiency using PERT and, the best duodenal pH to allow for the optimal efficacy of these extracts, but also the decontamination of the intestinal lumen, the supplementation of bile acids and, probably, the use of probiotics to attenuate intestinal inflammation in chronic pancreatitis patients[37]. Fat soluble vitamins and micronutrients, such as zinc and selenium, should be routinely assessed and administered whenever necessary[38].

Warnings regarding PERT

An appropriate clinical response to PERT does not allow predicting a normal nutritional status in patients with chronic pancreatitis. Up to 2/3 of patients with an apparently good clinical response have some residual nutritional deficiency[39]. Crushing, chewing or holding the pancreatic extract capsules in the mouth may cause local irritation. The fine powder of the pancreatic enzymes may also be irritating to the nasal mucosa and the respiratory tract and can precipitate an asthma attack. Extremely high doses of pancreatic extracts have been associated with hyperuricemia and hyperuricosuria[40]. Submucosal

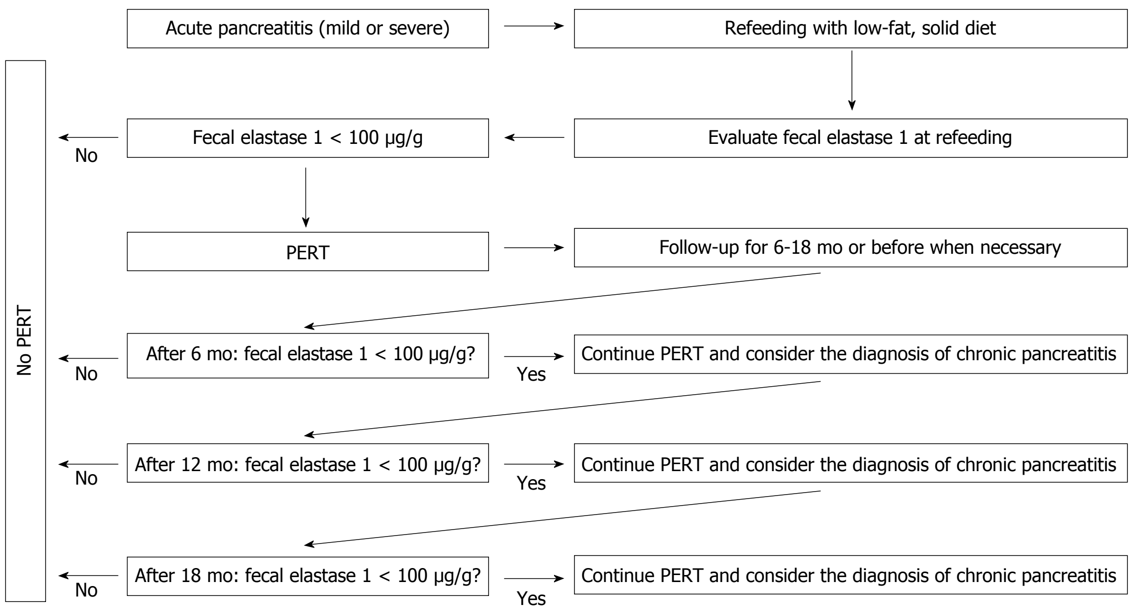

Figure 1 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients hospitalized for acute pancreatitis. PERT: Pancreatic enzyme replacement therapy.

strictures in the proximal colons of children with cystic fibrosis have been reported (“fibrosing colonopathy”), and it is now recommended that not more than 10000 units of lipase per kg of body weight per day be given to children[41]; to our knowledge, this complication has been never reported in adults[42].

RECOMMENDATION FOR SPECIFIC DISEASES

Acute pancreatitis

In Italy, there are approximately 20000 admissions per year for acute pancreatitis (AP)[43]. Acute pancreatitis is an inflammatory disease most commonly caused by gallstones or alcohol abuse, and is associated with significant morbidity and mortality[44]. Pathological values of fecal elastase-1 have been found in 12.0% of patients with AP (9.3% with mild and 2.7% with severe pancreatitis). Pathological fecal elastase-1 was not significantly related to sex, age or day of refeeding. Finally, only 4.0% of patients may have severe EPI (*i.e.*, fecal elastase-1 concentrations less than 100 μg/g). Thus, in selected cases (approximately 800 Italian AP patients per year), there is the need for enzyme supplementation during refeeding if the elastase-1 fecal determination is clearly abnormal[45]. The suggestion is that these patients be monitored for EPI for at least 6-18 mo and treated with oral pancreatic enzymes at a dosage of 40000-50000 U per meal and 25000 U per snack unless otherwise indicated[27] (Figure 1).

Chronic pancreatitis

The greatest benefit of PERT is in the patients who excrete more than 15 g of fecal fat per day or have weight loss[46,47]. However, German and Spanish guidelines treat patients with a daily fecal fat output < 15 g in the presence of symptoms of malabsorption (weight loss, osteopenia, loss of muscular mass)[9,31,48,49]. Alcohol should also be avoided to prevent additional impairment of the pancreatic exocrine function[50].

The initial dose of pancreatic enzymes should be 40000 units as a starting dose for a meal and 20000 units for a snack[9,31,48,51].

Increasing doses of PERT are recommended in non-responder patients[9,48,51]. Furthermore, acid suppression is also suggested to ensure optimal enzymatic delivery into the duodenum, despite the lack of clinical trials[52]. Moreover, as reported by Domínguez-Muñoz *et al*[53], gastric acid inhibition avoids bile acid precipitation and allows lipase release in the proximal gut. It has been shown that patients with EPI respond properly to PERT if bicarbonate secretion is preserved and/or gastric secretion reduced. Calcium and magnesium-containing antacids should be avoided as they produce soaps, precipitate with glycine conjugated bile salts in the intestine and worsen steatorrhea[54].

Lack of patient compliance may be a cause of treatment failure and can be discovered by measuring fecal chymotrypsin[55]. If chymotrypsin activity in the stool is low, the patient should be educated to take supplements during or just after meals[9,56]. Intestinal bacterial overgrowth, found in up to 40% of the patients with chronic pancreatitis[57,58], intestinal giardiasis or other intestinal malabsorption disorders, should be ruled out in non-responder patients.

Parameters to be used for the assessment of therapy include clinical improvement/normalization of nutritional parameters and clinical symptoms[9,24]. In non-responder patients, laboratory methods for assessing fat absorption (CFA, C-13 mixed triglyceride breath test) may be used. Fat soluble vitamin deficiency should be

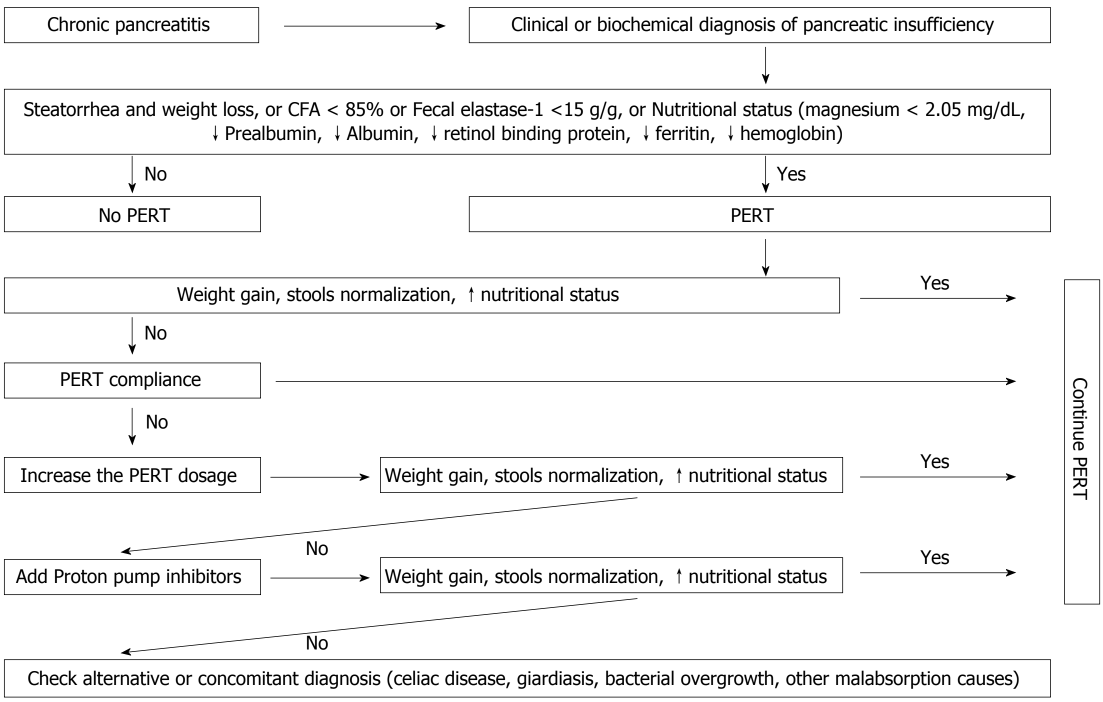

Figure 2 Algorithm for monitoring and treating exocrine pancreatic insufficiency. Algorithm for monitoring and treating exocrine pancreatic insufficiency summarized from Italian[9], German[47] and Spanish[31] guidelines, and a synopsis of the guidelines[51]. CFA: Coefficient of fat absorption; FE1: Fecal elastase-1; PERT: Pancreatic enzymes replacement therapy; PPI: Proton pump inhibitor.

corrected parenterally[9].

Before starting PERT, evaluation of the fasting glucose levels and quantification of the malabsorption is suggested, if possible. Moreover, determining glucose fasting levels during the first 1-2 wk of treatment is also suggested[51]. An algorithm for PERT in chronic pancreatitis patients is summarized in Figure 2.

Unresectable pancreatic ductal adenocarcinoma

The prevalence of EPI is high but of moderate degree in the majority of cases; it has been reported that 65% of pancreatic cancer patients have fat malabsorption, and 50% protein malabsorption[59,60]. The causes of the EPI are mainly related to the obstruction of and/or the loss of the pancreatic parenchyma[61]. Thus, the most important predictors of the onset of EPI malabsorption in pancreatic cancer patients are the site of the tumor in the pancreatic head, the tumor replacing at least 90% of the normal pancreatic tissue and main duct obstruction[62-64].

Even if the most widely accepted prognostic factors in unresectable pancreatic carcinoma are the presence of metastases and the value of CA 19-9 at presentation[65,66], the prognostic factor "weight loss" has received particular attention from the "Eastern Cooperative Oncology Group" study[66] and in this study the weight loss ranged from 30% in patients with non-Hodgkin's lymphoma to 87% in patients with gastric cancer; patients with pancreatic cancer showed weight loss in 65% of cases and it correlates with worsening of the performance status even if this factor was not a negative prognostic factor for survival[66]. In contrast, a more recent retrospective study found a direct relationship between the percentage of weight loss and the risk of death, with a value greater than 7 times the expected value when the decrease exceeded 10%[67] and these data were confirmed by a retrospective study regarding 58 patients with unresectable pancreatic carcinomas showing that a phase angle of less than 5 degrees was a negative prognostic factor[26] and by a prospective non-randomized study enrolling 194 patients with unresectable advanced pancreatic cancer showing that a value of fecal elastase-1 less than 20 μg/g was a negative prognostic factor for survival. Of interest, a value of fecal elastase-1 of less than 20 μg/g and extremely severe pancreatic insufficiency were found more frequently in the group of patients with tumors in the head of the pancreas[68].

The main question is whether replacement therapy with pancreatic enzymes and nutritional therapy have a positive impact on the quality of life and survival in patients with advanced pancreatic cancer. Pancreatic enzyme replacement therapy can partially prevent weight loss in patients with unresectable tumors of the pancreatic head, at least in the period before biliary endoprosthesis placement[69]. Two different phase Ⅱ studies have shown that, in patients with advanced pancreatic cancer, having a weight loss of more than 5% in the previous 4 wk and a body mass index of less than 19, parenteral nutrition improved all nutritional parameters, as evaluated by the bioelectrical impedance without, however, reaching normality[70,71].

The algorithm for monitoring EPI and malnutrition in unresectable pancreatic ductal adenocarcinoma patients is reported in Figure 3. Of course, appropriate amounts of pancreatic extracts should be administered during each

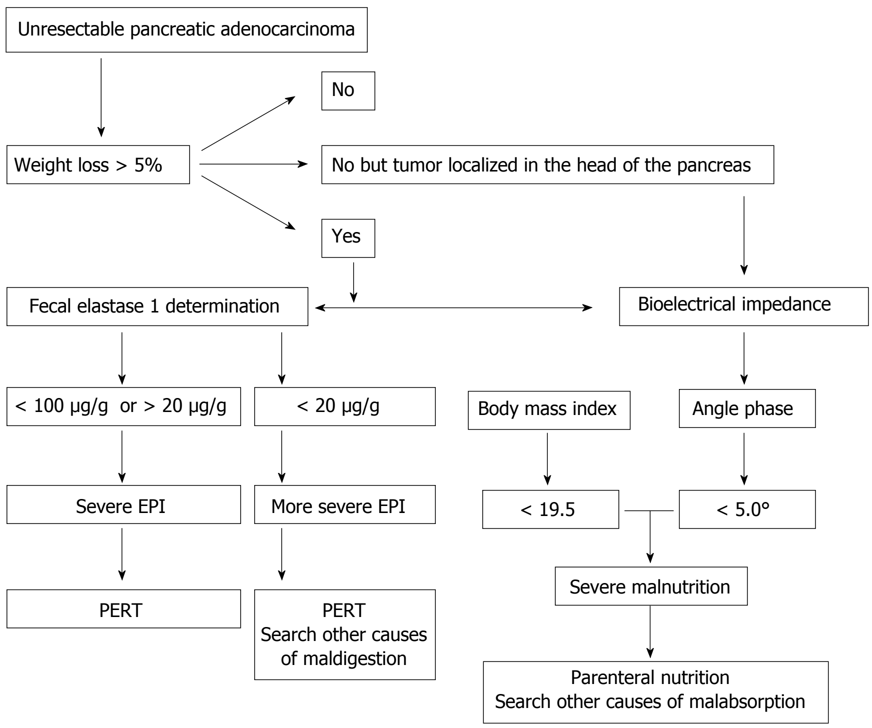

Figure 3 Algorithm for monitoring and treating exocrine pancreatic insufficiency and malnutrition in unresectable pancreatic ductal adenocarcinoma patients. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.

Table 4 Fecal elastase 1 concentrations in type 1 and type 2 diabetes mellitus *n* (%)

Ref.	Type 1 DM			Type 2 DM		
	Overall	FE-1 (100-200 μg/g)	FE-1 (< 100 μg/g)	Overall	FE-1 (100-200 μg/g)	FE-1 (< 100 μg/g)
Hardt *et al*[80]	322	73 (23)	92 (28)	697	108 (15)	138 (20)
Vesterhus *et al*[81]	140	10 (7)	16 (11)	63	2 (3)	6 (9)
Larger *et al*[82]	195	28 (14)	38 (19)	472	35 (7)	50 (10)
Icks *et al*[83]	112	22 (20)	29 (26)			
Cavalot *et al*[84]	37	17 (46)	4 (11)			
Rathmann *et al*[85]				544	100 (18)	65 (12)
Nunes *et al*[86]				42	6 (14)	9 (21)
Yilmaztepe *et al*[87]				32	9 (28)	1 (3)
Overall	837	158 (18.9)	188 (22.5)	1933	275 (14.2)	283 (14.6)

DM: Diabetes mellitus; FE-1: Fecal elastase-1.

meal (40000-50000 U of lipase) and per snack (25000 U).

Diabetes mellitus

Exocrine pancreatic insufficiency was demonstrated in approximately 50% of patients with insulin-dependent diabetes, and in 30%-50% of those with non insulin-dependent diabetes[72-78]. Nine prospective reports evaluated EPI by means of fecal elastase-1 estimation in patients with either type-1 or type-2 diabetes[79-87] (Table 4) and included 2770 diabetic patients, 837 of them (30%) with type-1, and the remaining 1933 with type-2 diabetes mellitus (DM). Overall, fecal elastase-1 concentrations were abnormal (*i.e.*, < 200 μg/g) in 904 patients (32.6%) and the impairment was mild (*i.e.*, fecal elastase-1 > 100 but < 200 μg/g) in 439 patients (15.8%, overall); severe EPI (< 100 μg/g) was documented in 465 (16.8%). Of the 904 diabetics with abnormal fecal elastase-1, exocrine impairment was mild in 48.9%, and severe in 51.4%. The prevalence of EPI differed slightly between type 1 and type 2 DM. Abnormal (< 200 μ/g) fecal elastase-1 concentrations were found in 346 (41.3%) of 837 type 1 diabetic patients, and in 558 (28.9%) of 1933 type 2 diabetic patients, a 12.4% difference in prevalence rates. More patients with type 1 DM (188 of 837, 22.5%) had signs of severe EPI, as compared to the 14.3% rate (277 of 1933) in type 2 DM. Exocrine pancreatic insufficiency is usually only of a mild to a moderate degree, and will not lead to clinically overt steatorrhea in the majority of diabetics. Thus, the clinical relevance of EPI and the role of functional tests in these patients are questionable. However, patients with DM frequently suffer from a wide range of abdominal complaints which contribute to impairment of the quality of life[88]. Although data are controversial, at least some of these symptoms may be attributable in

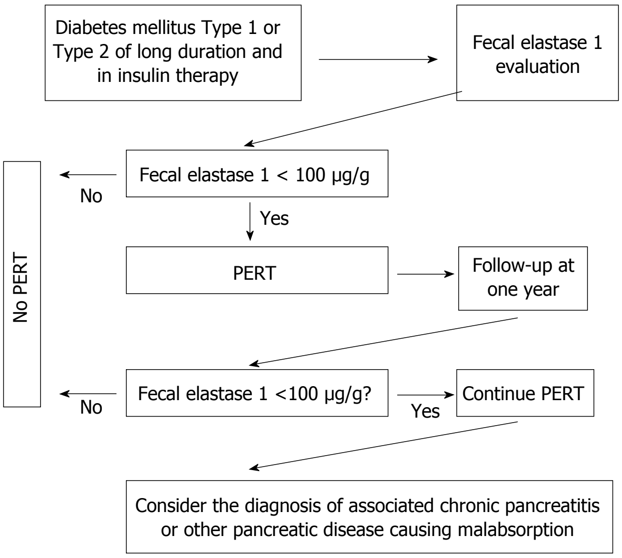

Figure 4 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients with diabetes mellitus. PERT: Pancreatic enzymes replacement therapy.

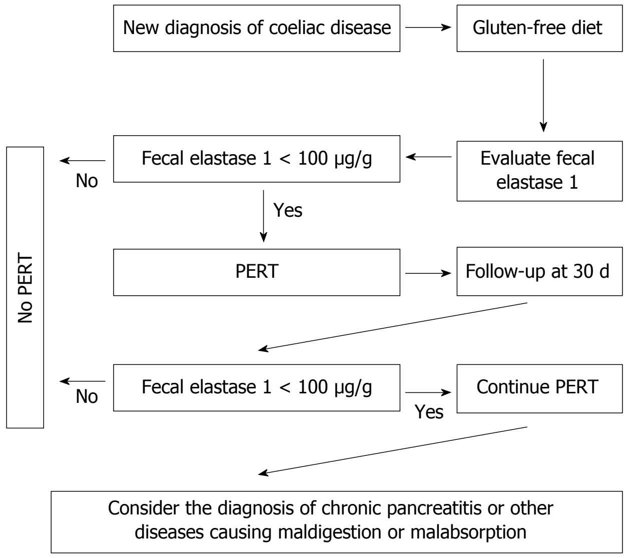

Figure 5 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients with celiac disease. PERT: Pancreatic enzymes replacement therapy.

part to EPI (mild to moderate) and might respond to enzyme treatment[81,89-94]. Thus, pancreatic tests should be part of the diagnostic work-up in patients with symptoms and do not respond to simple therapeutic measures. As reported in Figure 4, patients with fecal elastase-1 < 100 μg/g should be given pancreatic enzymes in adequate daily doses (40000-50000 U of lipase) administered during meals. Treatment improves symptoms significantly, the supply of soluble fat vitamins is normalized, and the risk of osteoporosis is reduced. Enzyme replacement therapy might have an impact on glucose metabolism since it can reduce the insulin requirement and contribute to improved control of the glucose metabolism, but the evidence is contradictory[93,94] as improvement of glucose metabolism was not seen in all studies[95,96].

Celiac disease

The prevalence of adult celiac disease (CD) in the general population is reported to be 1%-2%[96-99]; diarrhea remains a common presenting symptom[100] and it is usually attributed to continued gluten ingestion; however, other causes of chronic diarrhea in patients who are compliant with their gluten-free diet exist, and one of them is exocrine pancreatic insufficiency. Using a secretin test, it has been found a mild reduction in the pancreatic secretion of bicarbonates and pancreatic enzymes (especially lipase) in untreated celiac patients these alterations revert to normal after going on a gluten-free diet; mild pancreatic insufficiency is present in about 40% of untreated CD patients and severe pancreatic insufficiency in 10%[101,102]. More recently, other authors using tubeless test, such as fecal chymotrypsin or elastase 1 determination and the C mixed-triglyceride breath test, confirmed that pancreatic insufficiency in untreated CD patients in percentages ranging from 11.4% to 56.2%[103-107].

It has also been suggested exocrine pancreatic function impairment may be related to the degree of mucosal villous atrophy and that the level of fecal elastase may improve once the mucosa has recovered after an appropriate gluten-free diet[104,108]. In addition, it seems that pancreatic insufficiency does not depend on nutritional status[105]. Regarding the use of PERT in these patients, the data come from a double blind randomized study carried out on children showing that pancreatic enzyme therapy is certainly useful in the first 30 d after the diagnosis of CD[106]. In fact, after 30 d of a gluten-free diet associated with pancreatic extracts, body weight significantly increases with respect to patients treated with only a gluten-free diet. Similar results were obtained in a longitudinal study[109]. The conclusion is that pancreatic enzyme therapy is certainly useful in the first 30 d after the diagnosis of CD and that enzyme supplementation may possibly be discontinued as symptoms improve and fecal elastase-1 concentrations normalize. The dosage of pancreatic extracts should be 40000-50000 U per meal and 25000 U per snack. In CD patients who continue to experience clinical steatorrhea despite being on a gluten-free diet, a search for possible exocrine pancreatic insufficiency must be carried out[110]. In addition, we should bear in mind that, in adult CD patients have a risk developing chronic pancreatitis more than 3 times as compared to general population and there is also and increased need for PERT[111]. An algorithm for PERT in CD is reported in Figure 5.

Inflammatory bowel diseases

Crohn's disease: About 35% of the patients with Crohn's disease have an impaired exocrine pancreatic function[112] and no relationship are present between exocrine pancreatic insufficiency and age or nutritional status. Interestingly, patients having steatorrhea had a defect of lipase output ranging from 10% to 67% and, especially in this

latter group of patients, the use of PERT was hypothesized. In patients with Crohn's disease, enzyme activities were not correlated to the duration of disease or to the extent or localization of a previous bowel resection[113]. The lowest enzyme values were found in patients with the most extensive bowel involvement, and they were significantly lower than in patients with disease confined to the terminal ileum. Thus, the factors related to the impaired pancreatic function in Crohn's disease seem to be disease activity, and the localization and extent of the disease. Finally, patients with Crohn's disease may have an autoimmune involvement of the pancreatic gland and those having positive serum pancreatic autoantibodies may also have impaired exocrine pancreatic function more frequently[114]. However, we have no evidence that PERT can be utilized in patients with Crohn's disease and exocrine pancreatic insufficiency to improve the maldigestion present in these patients.

Ulcerative colitis: Pancreatic exocrine insufficiency, assessed using a secretin-cerulein test, may be present in about 40%-50% of patients with ulcerative colitis[112,115,116] especially during a active phase of the disease and the majority of patients with pancreatic insufficiency had active disease with loose stools; thus, the reduced fecal elastase-1 concentration could have been due to dilution of the enzyme and not to pancreatic involvement. In addition, in those patients who were also studied during the remission phase of the disease and had a solid stool, the fecal elastase-1 concentration became normal[112]. More recently, the possibility of autoimmune pancreatitis associated with ulcerative colitis has been reported. Thus, it is possible that only a small number of ulcerative colitis patients having severe pancreatitis insufficiency due to autoimmune pancreatitis may benefit from PERT.

Gastric surgery

Exocrine pancreatic insufficiency is a common clinical problem after gastric surgery[117]. The side effects of gastric resections, in particular total gastrectomy, include diarrhea, anorexia, weight loss and EPI that are responsible for a global status of malnutrition, malabsorption and maldigestion[118]. Malnutrition is considered one of the major complications after gastric surgery for gastric cancer[119] and EPI contributes to the pathogenesis of global malnutrition. After gastric surgery, EPI can result from various causes, such as a deficient trituration of nutrients, altered gastric emptying, alteration of pancreatic denervation and post-cibal asynchrony[120,121].

Any surgical procedure, such as total or subtotal gastrectomy, total or subtotal pancreatectomy, with or without duodenal resection (*e.g.*, in the context of a Whipple procedure), causing distortion in the anatomo-physiology of digestion can be responsible for EPI[122-127]. Several events can be considered as being responsible for EPI after gastrectomy. Alterations of gastric relaxation due to the absence of nervous gastric reflexes; the absence of nervous gastric stimulation responsible for pancreatic secretion caused by the lack of fundus relaxation and the reduction of exocrine pancreatic secretion due to the absence of cholecystokinin after intestinal resection. Rapid gastric emptying and asynchrony between gastric emptying and biliopancreatic secretion due to new tracts of various reconstructions, bacterial overgrowth after gastrectomy, extensive denervation of the pancreas due to lymph node dissection and truncal vagotomy are the most frequent alterations involved in the pathogenesis of EPI[123,128,129]. The latter has been shown to cause mild to moderate EPI by itself[130,131]. In 1996, Friess *et al*[123] demonstrated that 100% of patients develop severe primary EPI three mo after a total gastrectomy. Chymotrypsin and trypsin were the most severely deficient enzymes after gastric surgery, with a decreased production of up to 91% three mo after surgery. Low levels of gastrin and postprandial pancreatic polypeptides, and high levels of cholecystokinin were also reported[123]. Exocrine pancreatic insufficiency is reported in both total and partial gastrectomy; Büchler *et al*[127] demonstrated that the pancreolauryl test was pathological in 47%-64% of patients after Billroth-I surgery and in 64%-70% after Billroth-Ⅱ surgery. On the contrary, Heptner *et al*[124] reported EPI after gastric resection in only 30% of patients, even if the pancreolauryl test was abnormal in 90% of these patients. Armbrecht *et al*[132] conducted a double-blind, crossover study of 15 patients who underwent surgery for gastric cancer (total gastrectomy) and compared PERT with a placebo. The authors concluded that PERT reduced massive steatorrhea and improved stool consistency after total gastrectomy. Nevertheless, Bragelmann and coworkers reported an overall improvement in abdominal symptoms, fecal frequency and fecal consistency when following 52 institutionalized patients with a fecal fat output greater than or equal to 14 g/d after gastric resection for cancer, but no differences were found regarding body mass index, bowel habits or fat malabsorption[133]. Interestingly, Huddy and coworkers found that EPI contributes to postoperative morbidity after an esophagectomy, and that these patients can benefit from PERT[134].

The main goal of the therapy in patients suffering from EPI is to reverse all the secondary events caused by enzyme deficiency (Figure 6). Therapeutic efficacy is closely connected with two important aspects: time and dosage of the pancreatic enzymes administered, and dietary changes[135]. Nutritional changes should include a high carbohydrate diet, with normal fat and medium-chain triglycerides[136]. It is also recommended that a personalized diet be created after major gastrointestinal surgery in order to prevent weight loss, anorexia, inflammation and changes in homeostasis[53]. Following a total gastrectomy and in patients receiving therapy with proton pump inhibitors (PPIs) for some reason, unprotected pancreatin powder is preferred[120]. In addition to dietary changes it is mandatory to resort to PERT, orally ingested, during meals. The dose must be adapted to the meal and should not be less than 40000-50000 U of lipase per meal[135]. For partial gastric resection, patients receiving

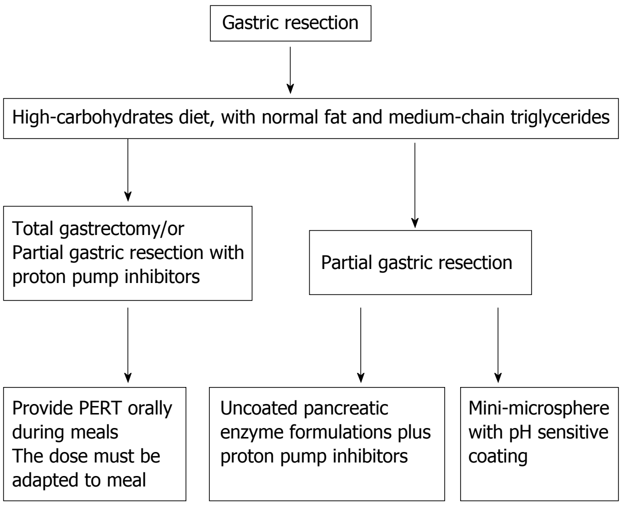

Figure 6 Algorithm for treating exocrine pancreatic insufficiency in patients who undergo gastric resection. PERT: Pancreatic enzymes replacement therapy.

uncoated pancreatic enzyme formulations should also require simultaneously administered proton pump inhibitors[53] since lipase is irreversibly deactivated by gastric acid. However, the administration of PPIs can improve fat digestion even in patients who do not benefit from PERT[53]. Several authors agree with the need for liposoluble vitamin supplementation, especially for patients with severe EPI[135].

Pancreatic resections

Suggestions on this topic are based on a consensus reached by the experts and are not fully based on data coming from literature. Partial or total pancreatectomy (TP) is frequently associated with EPI. In this setting, PERT is essential for maintaining adequate digestion. In a TP, the removal of the entire pancreatic parenchyma produces inevitable exocrine failure while, in a partial pancreatectomy, the severity of EPI depends on both the underlying disease, the preoperative pancreatic function, and the extent and type of the resection. Most importantly, any pancreatic neoplasm can be associated with chronic obstructive pancreatitis (focal or extended) which might affect pancreatic function/secretion, leading to EPI before any type of resection.

Extent and type of the resection

Pancreaticoduodenectomy: Anatomical changes secondary to reconstruction after a pancreaticoduodenectomy (PD) lead to important physiological alterations which frequently correlate with the severity of postoperative EPI. A PD (either Whipple or pylorus-preserving) is associated with several and complex patho-physiological events such as: (1) disturbance of gastric fundus relaxation caused by the disappearance of antro-fundic and duodeno-fundic reflexes; (2) the absence of neurally stimulated pancreatic excretion caused by the lack of fundus relaxation; (3) the reduction of cholecystokinin-mediated stimulation of pancreatic secretion secondary to duodenal resection; (4) large and hard to digest nutrient particles reaching the jejunal lumen due to resection of the distal stomach (Whipple procedure); (5) reduction in exocrine pancreatic secretion due to pancreatic head resection; and (6) asynchrony between the gastric emptying of nutrients and bilio-pancreatic secretion as a result of anatomical reconstruction[122,128,137,138].

For these reasons, every patient who is candidate for a PD should be considered at increased risk for EPI regardless of the underlying disease 128138. Therefore, it has been suggested that, after PD, PERT be given to all patients with pancreatic cancer, especially those with impending adjuvant therapy. Furthermore, it should be considered that pancreatic cancer is often associated with obstructive chronic pancreatitis, a preoperative risk factor for the development of EPI by itself[128,139]. The development of pancreatic insufficiency after PD could also be related to the different techniques used for the pancreatic anastomosis[117,140,141].

Distal pancreatectomy: Distal pancreatectomy (DP) is the procedure of choice for treating lesions affecting the body-tail of the gland. A DP may affect pancreatic exocrine function depending on the amount of normal tissue removed[142,143]. Based on these data, permanent postoperative EPI, as a result of parenchymal loss from pancreatic resection, was not observed and these conclusions have been subsequently confirmed[139].

Atypical resections: Middle pancreatectomy and enucleation: Atypical resections are usually performed for benign or borderline pancreatic tumors, such as small (< 2 cm) pancreatic neuroendocrine tumors, cystic papillary tumors, low grade intraductal papillary mucinous neoplasms and serous cystic adenomas. Enucleation is usually performed for tumors smaller than 2 cm located in any part of the pancreas but sufficiently far from the main pancreatic duct. A MP is indicated for neoplasm in the neck of the pancreas which could not be safely enucleated[139,144-146]. Using the ^{13}C-mixed triglyceride breath test (normal test > 5%), it has been found an EPI rate of 5% after a median follow up of 71 mo[147]. In addition, Crippa *et al*[148], after a median follow-up of 54 mo, observed a rate of clinical EPI of 5% in a cohort of 100 patients who had undergone MP for benign or borderline tumors. The authors compared this result with an EPI rate of 15.6% in patients who underwent an extended left pancreatectomy (at the right side of the superior mesenteric vein), the alternative surgical procedure to MP for lesions located in the pancreatic neck. These data are consistent with those of others[149,150].

Treatment of EPI in pancreatic resection

It has been reported that in patients undergoing a pylorus-preserving pancreaticoduodenectomy for pancreatic neoplasia, gastro-protected microspheres were less effective than those in patients who had undergone a classic Whipple technique[151] probably because microspheres are retained in the stomach. One of the few randomized

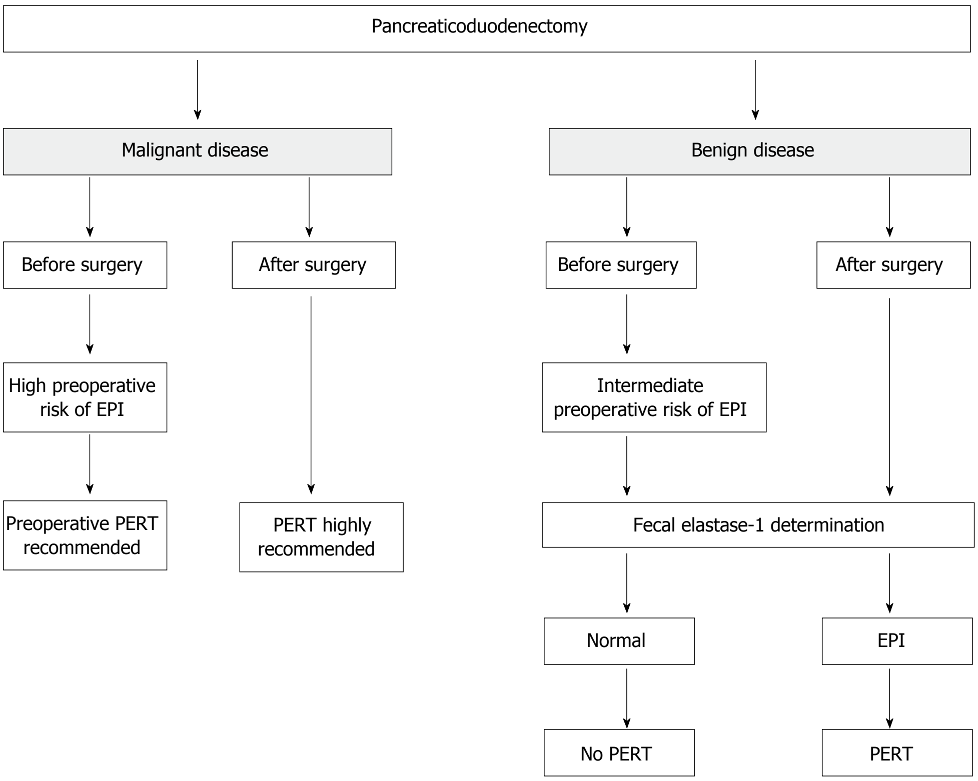

Figure 7 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who receive pancreaticoduodenectomy. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzyme replacement therapy.

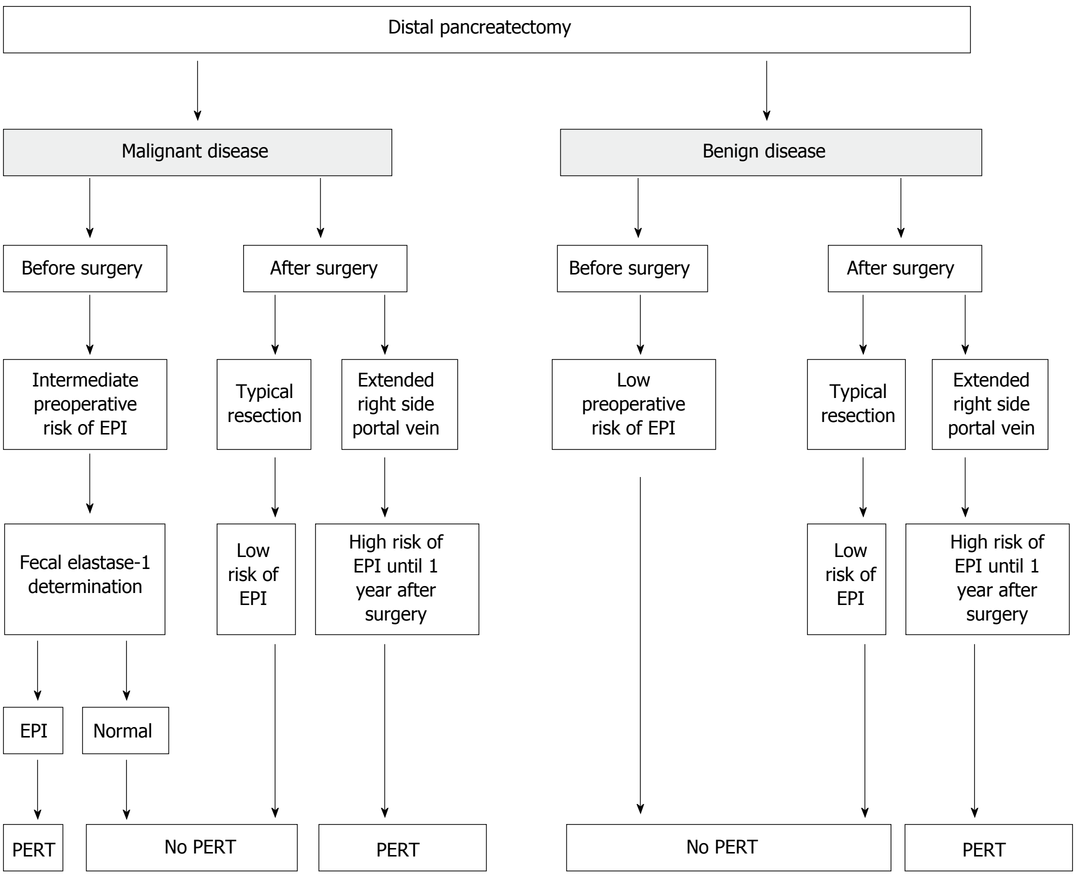

Figure 8 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who undergo distal pancreatectomy. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.

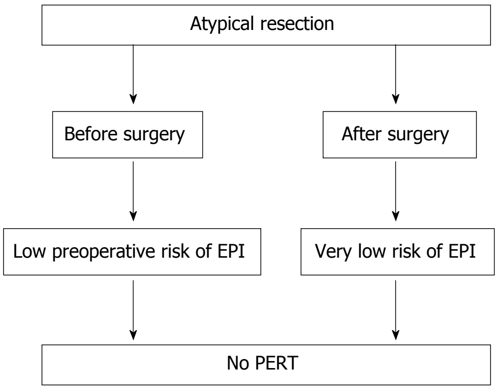

Figure 9 Algorithm for monitoring and treating exocrine pancreatic insufficiency in patients who undergo atypical resection of the pancreas. EPI: Exocrine pancreatic insufficiency; PERT: Pancreatic enzymes replacement therapy.

studies explaining the efficacy of pancreatic extracts for the control of malabsorption was carried out on a small group of patients with chronic pancreatitis who had undergone a pancreatic resection with longitudinal pancreaticojejunostomy[152] and showed that treatment with pancreatic extracts ameliorated not only the nitrogen balance but also the fat and protein absorption. Another randomized controlled double-blinded crossover study explored the comparative efficacy of two pancreatin preparations of gastroprotected microspheres with different doses in pancreatectomized patients having chronic pancreatitis[153]. All patients were stabilized before enrollment in the study with a standard dose of pancreatic extracts. After this stabilization period, 56% of the patients still had a fecal fat excretion greater than 7 g/d, and 38% greater than 15 g/d. The results demonstrated that there was a significant relationship between fecal fat excretion, fecal volume and evacuation frequency but there was no relationship between fecal fat excretion, and abdominal pain or malabsorption. Both the pancreatin standard dose and the elevated dose demonstrated equal efficacy; in pancreatectomized patients, high dose pancreatic extracts significantly reduced the number of capsules needed per day with a better compliance to substitutive therapy. From the clinical point of view, pancreatic enzyme replacement therapy needs to be routinely considered and based on pragmatic clinical evaluation of the patient[22,38,63]. The suggested algorithms for PERT in patients undergoing surgery, according to the type of pancreatic resection, are reported in Figures 7-9, taking into consideration that the dosage should be no less than 40000-50000 U of lipase per meal and 25000 per snack.

CONCLUSION

We should point out that there is a paucity of information regarding some areas of managing EPI there is a lack of good quality of literature. Finally, studies on the economic aspects of this treatment with the different formulations commercially available are also necessary; it has been calculated that the treatment of chronic pancreatitis-related EPI with pancreatin minimicrospheres is cost-effective according to a survey of Polish patients but it is necessary that these results also be evaluated for the Italian National Health System[154].

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REVIEW

MicroRNAs as tools to predict glucocorticoid response in inflammatory bowel diseases

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Abstract

In spite of the introduction in therapy of highly effective biological agents, glucocorticoids (GCs) are still employed to induce remission in moderate to severe inflammatory bowel diseases (IBD), but considerable inter-individual differences in their efficacy and side effects have been reported. The effectiveness of these drugs is indeed very variable and side effects, particularly severe in pediatric patients, are common and often unpredictable: the understanding of the complex gene regulation mediated by GCs could shed light on the causes of this variability. In this context, microRNAs (miRNAs) represent a new and promising field of research. miRNAs are small non-coding RNA molecules that suppress gene expression at post-transcriptional level, and are fine-tuning regulators of diverse biological processes, including the development and function of the immune system, apoptosis, metabolism and inflammation. Emerging data have implicated the deregulated expression of certain miRNA networks in the pathogenesis of autoimmune and inflammatory diseases, such as IBD. There is a great interest in the identification of the role of miRNAs in the modulation of pharmacological response; however, the association between miRNA and GC response in patients with IBD has not yet been evaluated in a prospective clinical study. The identification of miRNAs differently expressed as a consequence of GC treatment in comparison to diagnosis, represents an important innovative approach that could be translated into clinical practice. In this review we highlight the altered regulation of proteins involved in GC molecular mechanism by miRNAs, and their potential role as molecular markers useful for predicting in advance GC response.

Key words: Glucocorticoids; Inflammatory bowel diseases; MicroRNA; Molecular markers; Pharmacogenomics

Core tip: Studies on microRNAs (miRNAs) and pharmacogenomics represent a promising investigation topic that could increase the understanding of the pharmacology of steroids in inflammatory bowel diseases (IBDs) and possibly in other diseases. A number of studies have shown that glucocorticoids (GCs) can modify the expression profiles of different miRNAs, however, the obtained results have been highly variable, and to date it is not possible to recognize a specific miRNA pattern regulated by GCs. Moreover, existing studies employed techniques based on the use of reverse transcription quantitative polymerase chain reaction and microarrays, through the analysis and quantification of already known miRNAs. Using next generation sequencing technologies, it could be possible to detect novel, still unrecognised miRNAs, and identify new miRNA isoforms (iso-miRs) as well. This innovative approach could be a valuable tool for a better understanding of the role

of miRNAs to predict steroid response in IBDs. In the future, the increased availability and the reduced costs of RNA profiling should enable the clinicians to stratify patients on specific miRNA biomarkers before starting GC treatment.

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INTRODUCTION

To date, a curative pharmacological therapy for inflammatory bowel diseases (IBD) does not exist and the therapeutic approach is mainly aimed at controlling inflammation, with drugs capable of inducing and maintaining remission. Despite the introduction in therapy of highly effective biological agents, in IBD patients with moderate to severe disease glucocorticoids (GCs) are effective in inducing remission and are still considered the standard for treatment[1]. In spite of the large clinical use, the benefits of these agents are often narrowed by high inter-individual variability. Given the high incidence of suboptimal response, associated with a significant number of side effects, the identification of subjects that are most likely to respond poorly to these agents is extremely important. However, the mechanisms of this variability are scarcely understood and there is presently no means to predict the response in advance[2-5]; in this context, microRNAs (miRNAs) represent a new and promising field of research.

miRNAs are small (18-24 nucleotides) non-coding RNAs, which bind the 3'UTRs and the coding exons of their target genes and inhibit gene expression[6] either by messenger RNA (mRNA) cleavage (most common in plants) or by translational repression (most common in metazoan)[7,8]. According to the miRNA database miRBase, 1872 precursors and 2578 human mature miRNA sequences have been published (http://www.mirbase.org[9,10]) and we are only on the verge of understanding their physiological impact on gene regulation. A single miRNA can regulate a multitude of mRNAs (approximately 200), and each mRNA can be regulated by multiple miRNAs[11,12]; overall, it is predicted that protein production for at least 20% of all human genes is regulated by miRNAs[13,14].

By affecting gene regulation, miRNAs are likely to be implicated in the control of diverse biological processes, such as cellular proliferation and apoptosis[10,15-17], stem cell differentiation[15,18-20], and organ development and morphogenesis[21,22]; in addition a strong association between miRNA expression dysregulation and induction of cancer has been shown[23-26]. Moreover, miRNAs have important regulatory roles in the innate and adaptive immune system[27-29], and characteristic miRNA expression profiles have been demonstrated even in IBD[30-33].

There is a lot of interest in identifying the role of miRNAs in the modulation of drug response[34], but studies about this topic are still very limited, and the possible correlation between miRNAs expression and variability on GC response in IBD patients has not yet been examined. A better knowledge of miRNAs role could lead to their use as biomarkers for IBD, and consequently, to the development of new strategies for therapy personalization in these diseases.

This review tries to highlight the altered regulation of proteins involved in GC molecular mechanism by miRNAs in different diseases and *in vitro* models, and their potential role as molecular markers useful for predicting in advance GC response.

GLUCOCORTICOIDS IN INFLAMMATORY BOWEL DISEASES

GCs are effective inhibitors of cytokine secretion and T-cell activation, and are consequently largely employed in different inflammatory conditions, including IBD. Despite the introduction of novel therapies, these agents are still currently used for induction of remission in moderate to severe IBDs, however, a wide variability in response to these agents is evident and, in these diseases, GC resistance or dependence is particularly frequent. Among the adult IBD population, a prospective analysis has described the 1-year outcome in patients with Crohn's disease (CD) treated with a first oral prednisone course (40-60 mg/d) and tapering to a maintenance dose of 10-15 mg/d[35]. Prolonged steroid response was obtained in 44% of patients, 36% of subjects were steroid dependent while 20% of subjects did not respond and were steroid resistant; a high frequency of surgery was reported within 1 mo after steroid treatment. Similar results have been obtained in a retrospective American study: immediate outcomes for CD and ulcerative colitis (UC), respectively, were complete remission in 58% and 54% of cases, partial remission in 26% and 30%, resistance in 16% of patients[36]. In paediatric IBD patients, clinical reports have shown that up to 90% of subjects has a rapid improvement of symptoms when prednisone treatment is given; however, after 1 year, only 55% of patients were still in remission and were considered steroid responsive. In around 38% of patients, steroid therapy could not be discontinued as patients experienced an increase of disease activity when the dose was reduced (steroid dependent)[37].

Demographic and/or clinical markers[36,38,39] have been evaluated and related with this variability in GC response, but results have not been consistently replicated. Genetic and epigenetic markers are likely to complement clinical and demographic predictors: phenotypes resulting from genetic changes and regulation can markedly influence drug pharmacokinetics or alter drug efficacy and/or toxicity profiles. The identification of genetic biomarkers that can be useful for classifying the disease and help to improve therapy is paramount.

MOLECULAR MECHANISM OF GC ACTION

The effects of GCs are mediated by the glucocorticoid receptor (GR)-α, a member of the nuclear receptor superfamily of ligand-dependent transcription factors[40,41]. The human GR gene is encoded on chromosome 5q31.3 and consists of nine coding exons[42]. Alternative splicing of exon 9 generates two receptor isoforms, GR-α and GR-β[43-46]. GR-β is not able to bind GCs, resides constitutively in the nucleus of cells, has a longer half-life than GR-α, and does not transactivate GC-inducible reporter genes[47]. It has been suggested[48,49] that cell specific expression and function of GR isoforms may explain the tissue and individual selective actions of GCs.

The function of GR is conditioned by chaperone and co-chaperone proteins that form a molecular heterocomplex with the GR itself[50,51], required for proper ligand binding, receptor activation and transcription: abnormalities in proteins that make up the heterocomplex may contribute to altered GC responsiveness[52,53]. Several studies have demonstrated differences in the heterocomplex gene expression profiles in steroid resistant in comparison with responder patients, but it is not clear if this different expression is the cause of the variability in response or the consequence of GC treatment[54-59]. After GC binding and dissociation from heterocomplex proteins, the GR translocates into the nucleus; translocation is mediated by specific nuclear transport factors that belong to the importin β family of nuclear transporters, and in particular by importin 13[60]. The activated receptor then binds as homodimer two palindromic DNA-binding sites, the so-called glucocorticoid responsive elements (GREs), localized in the promoter region of target genes[61-63]. As a consequence of DNA binding, GCs can induce trans-activation and trans-repression processes: binding to positive GREs leads to activation of the transcription of anti-inflammatory [*e.g.*, interleukin 10 (IL-10), Annexin 1] as well as of regulator proteins involved in metabolic processes (*e.g.*, enzymes of gluconeogenesis)[64-66]. The second mechanism of GC action is trans-repression[67], which leads to a reduced expression of immune-regulatory and proinflammatory proteins such as cytokines [IL-1, IL-2, IL-6, tumor necrosis factor-α (TNF-α)] and prostaglandins[68], and is believed to be responsible for the majority of beneficial anti-inflammatory effects.

Steroid hormones can regulate gene expression post-transcriptionally, by destabilizing mRNAs[69]. In addition, these hormones can induce rapid non genomic effects within the cytoplasm; for example, they induce the release of Src kinase from the GR heterocomplex, resulting in lipocortin activation and inhibition of arachidonic acid release[70,71], and alter cytoplasmic ion content[72,73].

miRNAS AND GC RESPONSE

miRNA regulation by GCs

It has been demonstrated that activation of GR by GCs might induce or repress specific miRNAs in various target genes. The majority of studies have evaluated the effect of GCs on miRNA expression levels in tumor leukemic cells, during GC induced apoptosis[74].

Rainer *et al*[75] have correlated miRNA levels with expression data of their host genes in cell lines and clinical samples of children with acute lymphoblastic leukemia (ALL) undergoing systemic GC monotherapy. At least 5 miRNAs were significantly regulated by GC therapy. Importantly, the miR-15/16 cluster, which induces cell cycle arrest, was up-regulated by GCs in a subset of ALL patients and cell lines, consistent with the known apoptotic effect of GCs in immature lymphoblasts. Indeed, overexpression of miR-15b/16 increased GC sensitivity in leukemia cell lines whereas silencing miR-15b/16 with inhibitors decreased GC sensitivity *in vitro*.

Another study in a T-cell lymphoma cell line has shown that GC treatment repressed the expression of the miRNA cluster miR-17-92, which results in elevated protein expression of Bim, a proapoptotic member of the B-cell lymphoma-2 family (Bcl-2). Overexpression of miRNA cluster miR-17-92 decreased Bim induction, and attenuated GC mediated apoptosis, while cluster knockdown increased Bim induction and GC mediated apoptosis[76]. These findings suggest a novel mechanism that could contribute to the induction of lymphocyte apoptosis by GCs.

Harada *et al*[77] demonstrated that in the leukemic cell line RS4; 11 dexamethasone down-regulated miRNA levels; miR17HG was rapidly down-regulated, and chromatin immunoprecipitation demonstrated that the promoter is a target of GC transcriptional repression; in particular, the miR-17-92 cluster was identified as a prime target for dexamethasone induced repression. In the sensitive leukemia cell line SUP-B15, but not in the resistant line REH, dexamethasone reduced the expression of the mi-R17 family and concomitantly increased its target protein Bim. Up-regulation or inhibition of miR-17 resulted in a decrease and increase, respectively in Bim protein levels and in dexamethasone induced cytotoxicity. Down-regulation of miR-17 levels was observed in *ex vivo* patients' leukemia cells that underwent dexamethasone induced apoptosis[77].

Another recent study[78], by genome wide miRNA microarray on diagnostic bone marrow samples of ALL pediatric patients treated with GCs, identified a reduced expression of miR-355 as the most significant miRNA abnormality associated with poor outcome. Moreover, the authors demonstrated that exogenous expression of miR-355 in ALL cells increases sensitization to prednisolone-induced apoptosis. MAPK1 was identified as a target of miR-355, and the MEK/ERK inhibitor treatment increased GC induced cytotoxicity through the activation of Bim.

Smith *et al*[79] have demonstrated that miRNAs are repressed during GC induced apoptosis of primary rat thymocytes, and further demonstrated the repression

of the miRNA processing enzymes Dicer, Drosha and DGCR8/Pasha. Silencing of Dicer expression in two human leukemic lines significantly enhanced GC induced apoptosis, while overexpression of the GC-repressed miR-17-92 polycistron reduced apoptosis.

Among the few studies that have considered the effect of GCs on miRNA expression in non tumor cells, Ledderhose *et al*[80] in native and CD3/CD28 stimulated cells from healthy volunteers, demonstrated that miR-24 is expressed in human T cells, and expression is increased 1.7 fold upon stimulation. Hydrocortisone significantly enhanced by 3 fold the miRNA induction[80].

In human corneal fibroblast treated for 16 h with dexamethasone, genome microarray and microRNA analyses were used to evaluate gene and miRNA expression. In response to treatment with the steroid, 261 genes were up-regulated and 123 were down-regulated more than three-fold. Several miRNAs, including miR-16, miR-21 and miR-29C were up-regulated, whereas miR-100 was down-regulated by the steroid, suggesting a posttranscriptional control of gene expression through miRNAs[81].

Studies of the miRNAs profile on mucosal biopsies of patients with eosinophilic esophagitis, before and after successful treatment with GCs were conducted by Lu and collaborators[82]; of the 377 miRNA sequences examined, 32 miRNAs were significantly up-regulated and 4 down-regulated in the biopsies obtained before treatment compared to samples obtained after GC therapy. miR-214 was the most up-regulated (150 fold) and miR-146b-5b, 146a, 145, 142-3p and 21 were up-regulated by at least 10 fold.

Williams *et al*[83], using a highly sensitive reverse transcription-polymerase chain reaction, measured 277 miRNAs in airway biopsies obtained from normal subjects and mild asthmatic patients before and after one month twice dayly treatment with inhaled budesonide. No significant difference in miRNA expression was evident in the airway biopsies of normal and asthmatic subjects, and, despite improved lung function, no change in miRNAs expression was evident after one month budesonide treatment. However, a specific miRNA expression profile was observed in different cell types (alveolar epithelial cells, airway smooth muscle cells, alveolar macrophages, lung fibroblasts).

Finally, in a recent study[84], activated human CD4$^+$ T cells from healthy donors were exposed *in vitro* to 1 μmol/L of methylprednisolone and changes in miRNA and mRNA expression profiles were analyzed by microarrays; a number of steroid responsive genes and miRNAs were identified. Further studies with qPCR, flow cytometry and ELISA, demonstrated that methylprednisolone increased the expression of miR-98 and suppressed the levels of predicted targets, including the pro-inflammatory cytokine IL-13 and three TNF receptors FAS, FASL, and TNF receptor superfamily member 1B (TNFRSF1B): these data suggest that methylprednisolone acts through miR-98 to inhibit specific pro-inflammatory targets[84].

GR as miRNA target

The role of miRNAs in the regulation of the GR has been examined, indeed, computational studies showed that the 3' UTR of the GR is predicted to contain numerous seed regions recognized by a variety of miRNAs[85].

Using a combination of in silico prediction of miRNA binding sites, miRNA overexpression studies and mutagenesis of the GR 3'UTR, Vreugdenhil and collaborators[86] found that miR-18 and miR-124a bind GR mRNA and decrease GR activity in neuronal tissues. These miRNAs were tested for their ability to alter the translational activity of GR and reduce GR protein levels in cell cultures *in vitro*; miR-18 and miR-124a overexpression reduced GR protein levels and impaired the activation of the GR responsive gene glucocorticoid-induced leucine zipper (GILZ). In addition these authors have demonstrated by miRNA reporter assay that miR-124a is able to bind to the predicted seed region in the GR 3' UTR.

Ledderose *et al*[80] have investigated the role of miR-124 in the regulation of GR expression; these authors have studied the influence of the GR isoforms (the active isoform α, and the dominant negative non-ligand-binding isoform β) on GC effects in human T-cells, and found that, in patients with critical illness-related corticosteroid insufficiency, miR-124 specifically down-regulated GR-α: a slight increase of miR-124 and a reduction of GR-α was observed in patient T-cells compared to healthy controls. The authors suggested a novel miR-124-mediated mechanism in the down-regulation of GR-α in patients with critical illness-related corticosteroid insufficiency, that could explain, at least in part, GC resistance in this disease.

Tessel *et al*[87] have identified and characterized miR-130b as an important down-regulator of GR in GC-resistant multiple myeloma cell line: the overexpression of this miRNA was also associated with a decreased regulation of the downstream GC controlled gene GILZ, suggesting this mechanism as one of the possible causes of resistance to GCs.

miRNA involved in IBD

The pathophysiology of IBD is not yet clear, and genetic, epigenetic, infectious and immunological factors seem to play a role. It has been suggested that the gastrointestinal inflammation is the result of an altered activation of the immune system to a luminal factor, such as intestinal flora, in genetically predisposed subjects.

Among the many biological processes regulated by miRNAs, it is now accepted that these small non coding RNAs contribute to the maintenance of immunological homeostasis at mucosal sites[88,89]. The role of miRNAs in the pathogenesis of IBD has been thoroughly considered (see recent reviews[32,90,91]), and it has been suggested that these small non coding RNAs represent an important player in the complex interactions which results in IBD clinical features. Of particular interest is the observation that miRNA expression changes during tissue progres-

sion from normal to inflamed and varies according to the type and evolutionary stage of IBD[92]. Indeed, a number of studies have identified a specific differential expression of miRNAs in IBD and unique miRNA expression profiles for the different subtypes of IBDs, both in human tissues collected by colonoscopic biopsies and in peripheral blood samples, have been demonstrated[32,90,91].

It has been argued that genetic polymorphisms in miRNAs, as well as in miRNA target genes can affect their regulatory function and, consequently, the expression level of their target mRNAs. Most studies have described an association between SNPs in miRNA genes and human cancers[93-98], and only recently the association between mRNA related SNPs and the risk of IBD has been examined[99]. Bioinformatic approaches have been used to analyze the association between diseases-linked SNPs, miRNAs and mRNAs: SNP data derived from genome wide association studies that were correlated with miRNA, revealed a CD phenocode comprising rs11209026, rs7807268, rs254215, rs2542151 in miR-125, rs11805303 in miR-519, and rs6908425 in miR-181[30]. Of interest, miR-181, miR-519 and miR-119 could target mRNAs encoded by genes involved in the importin pathway, whereas miR-181 and miR-125 are potential regulators of components of inflammasome pathway. Both importin and inflammasome are involved also in GC molecular mechanism: importin is a nuclear transport protein responsible for the translocation of the complex GR-GC into the nucleus[2], and variants in inflammasome gene have been correlated with steroid resistance in pediatric IBD patients[100].

An association between rs3746444 in miR-499 and UC susceptibility has been observed in 170 Japanese patients: this SNP may alter the function or expression of miR-499, altering the regulation of target mRNAs related to inflammatory immune responses, and influencing the pathophysiological features of UC[101]. Of particular interest is the observation that the rs3746444 AG genotype was associated also with steroid dependence and refractory phenotype, whereas the rs3746444 AA genotype was inversely related to hospitalization time, steroid dependence, and refractory phenotype. In addition, the rs11614913 TT genotype held a significantly higher risk of refractory phenotype.

CONCLUSION

There is a lot of interest in identifying the role of miRNAs in the modulation of drug response, but studies about this topic are still very limited, and the possible correlation between miRNAs expression and variability in GC response in IBD patients has not yet been extensively examined. Studies about miRNAs and pharmacogenomics may represent a promising investigation topic that could increase the understanding of the pharmacology of steroids in IBDs and possibly in other diseases.

A number of studies have shown that GCs can modify the expression profile of different miRNAs, however, the obtained results have been highly variable. The differences observed can possibly be ascribed to the different tissues or cell lines analysed or different experimental protocols, and to date it is not possible to recognize a specific miRNA pattern regulated by GCs.

miRNA regulation by GCs in IBDs has never been analyzed in clinical prospective studies, in which patients are followed from diagnosis and throughout steroid therapy: the identification of miRNAs differently expressed as a consequence of GC treatment in comparison to diagnosis, could be an important innovative approach. This type of study design will reduce to the minimum the effect of confounding factors and results should be easier to translate into clinical practice.

Moreover, existing studies employ techniques based on the use of reverse transcription quantitative PCR and microarrays, based on the analysis and quantification of already known miRNAs. Using next generation sequencing technologies it should be possible to detect novel, still unrecognised miRNAs, and identify new miRNA isoforms (iso-miRs) as well.

In the future, the increased availability and the reduced costs of RNA profiling should enable the clinicians to stratify patients on specific miRNA biomarkers before starting GC treatment. This will allow the personalization of therapy, avoiding a treatment doomed to failure, increasing efficacy and reducing toxicity.

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REVIEW

Anti-angiogenic therapies for metastatic colorectal cancer: Current and future perspectives

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Abstract

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women in the United States, with about 142820 new cases and 50830 deaths expected in 2013. Metastatic disease (mCRC) remains a challenge for oncologists worldwide due to its potential comorbidities. Recently, chemotherapy regimens containing 5-fluorouracil, leucovorin, oxaliplatin and irinotecan combinations are a standard of care in the metastatic disease. Currently, biological therapies involving vascular endothelial growth factor and epidermal growth factor receptor pathways, such as bevacizumab and cetuximab, have emerged as good option for improving mCRC patient survival. Now, aflibercept plus standard chemotherapy has also been approved in second line regimen for mCRC patients. Our review will discuss novel biological drugs and their indications for mCRC patients and will bring future perspectives in this regard.

Key words: Colorectal cancer; Bevacizumab; Cetuximab; Aflibercept; FOLFOX; FOLFIRI

Core tip: Metastatic colorectal cancer is a very aggressive disease. However, recently developed chemotherapeutic protocols and targeted drugs have emerged as a valuable tool for treating this set of patients. Our manuscript brings the readers current trends and future perspectives in this field.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women in the United States, with about 142820 new cases and 50830 deaths expected in 2013[1]. In Europe, CRC represents the second most common cancer and leading cause of cancer death, in both genders combined[2]. Consequently, CRC is considered a prominent global health problem.

Usually, early CRC has no symptoms, and this is why screening is so important. Moreover, almost all symptoms (*i.e.*, change in bowel habits, general abdominal discomfort, weight loss with no apparent cause, constant tiredness) are not well specific. Consequently, CRC might be diagnosed when a patient has symptoms or as a result of a screening program[3]. Colonoscopy is the main diagnostic tool for primary screening due to its great benefit on either flexible sigmoidoscopy or guaiac fecal occult

blood test[4].

The 1- and 5-year relative survival rates for patients with CRC are respectively 83.2% and 64.3%, considering all stages. Additionally, ten years after diagnosis, survival continues to decline to 57.6%[5]. The most important problem remains disease relapse following surgery since, commonly, it is the cause of death in these patients[3]. This fact becomes relevant when we observe that when CRC are detected at a localized stage, the 5-year relative survival rate is 90.1% and, after disease involves adjacent organs or lymph nodes, the 5-year survival rate falls to 69.2%. Moreover, when cancer has spread to distant organs, the 5-year survival rate is 11.7%[5].

Many patients have metastatic disease (mCRC) initially not suitable for resection[6]. The majority of patients with mCRC cannot be cured, and the goals of chemotherapy for them are to prolong survival, improve quality of life and provide palliation, when applicable[7]. Over the past years, the outcome of these patients has been improved, with median survival reaching almost 24 mo[6,8].

The liver is the most common site of hematogenous metastasis in CRC, and its appearance is a frequent event for patients with CRC and remains a major cause of cancer-related death[9]. Approximately 25% of patients present synchronous liver metastasis at time of diagnosis, and another 25% of patients will develop liver metastases during the course of their disease, usually within a 2-year period after initial surgical treatment of their primary tumor[10]. The only potentially curative treatment for patients with liver metastasis is surgical resection, which results in a 5-year survival rate of 36%[11]. Nevertheless, 70% of these patients will suffer a relapse after resection of their hepatic metastasis, with the majority in the first 2 years after surgery and the remaining continuing to occur up to 10 years[12].

Over the past years, the development and incorporation of agents that target angiogenesis in clinical practice have led to improvements in the treatment of mCRC, with benefits in progression-free survival (PSF) and overall survival (OS) in these patients[13]. This paper aims to review the impact of known and new anti-angiogenic therapies for mCRC, especially those which target vascular endothelial growth factor (VEGF) pathways.

Angiogenesis and CRC-molecular mechanisms

Blood vessel formation comprises two main types: vasculogenesis and angiogenesis. During early embryonic development, vasculogenesis is the process responsible for the formation of the primary vasculature of the body, which consists in the formation of blood vessels from endothelial cell progenitors (*i.e.*, hemangioblasts)[14]. On the other hand, angiogenesis is a complex and highly regulated biological process that refers to the formation of new vascular segments. During this process, the combination of sprouting, splitting, and remodeling of the existing vessels occurs[15]. Physiologically angiogenesis occurs under tight regulation by a wide range of proangiogenic inducers, such as growth factors, chemokines, angiogenic enzymes, endothelial-specific receptors, and adhesion molecules as well as various antiangiogenic factors including angiostatin, endostatin, thrombospondin, canstatin, and pigment epithelium-derived factor[16]. As blood vessels are needed to supply nutrients and oxygen to tissues, angiogenesis plays an essential role in normal growth and development. Nevertheless, imbalances between the angiogenic mediators and inhibitors may result in the development of pathologies, as cancer[17].

In order to continue grow and metastasize, tumors need to continually acquire an adequate blood supply, which is accomplished by inducing angiogenesis[18]. Since Folkman recognized, in the early 1970s, the therapeutic potential for the inhibition of angiogenesis process in cancer, angiogenesis has been largely studied[19].

Figure 1 shows the main angiogenic mechanisms related to VEGF pathways. The VEGF family, which plays a critical role in tumor angiogenesis[20], includes six members: VEGF-A, -B, -C, -D, -E and placental growth factor (PIGF)[21]. VEGF-A, also known as VEGF, is the most important member and the major physiologic and pathologic mediator of angiogenic mechanism[20]. The *VEGF-A* gene, located on chromosome 6 (6p21.3), undergoes alternative splicing to yield mature isoforms of 121, 145, 165, 183, 189, and 206 amino acids[22-24]. *In vivo*, only three isoforms have been related to angiogenesis, $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$. The latter has been demonstrated to be a predominant isoform secreted by malignant and benign cells[25]. VEGF signals, mainly through VEGF receptor 2 (VEGFR-2) which is tightly expressed by endothelial cells, are involved in angiogenesis. VEGF binds to VEGF receptor 1 (VEGFR-1), with approximately 10 times the affinity of VEGFR-2 binding. However, its signal-transducing properties are extremely weak[26].

Most solid tumors present hypoxic regions as they grow and, thus, outweigh their blood supply. The results of the cellular adaptation to hypoxic microenvironment are aggressive disease, resistance to therapy, and decreased patient survival[27]. The transcription factor hypoxia-inducible factor-1 (HIF-1 or HIF) is the most important regulator of the hypoxic response, which upregulates expression of proteins involved in the regulation of several aspects of tumor biology, such as oxygen transport, iron metabolism, glycolysis, glucose transport, cell survival and proliferation, angiogenesis, invasion and metastasis[28,29]. VEGF is one of several proangiogenic factors directly activated by HIF-1 and acts to promote new blood vessel formation and thereby provide the re-establishment of oxygen and nutrient supply[27].

Paracrine mechanisms generated through VEGF production by tumor cells may also influence angiogenesis pathways. However, those cells cannot adequately respond to VEGF directly since they do not have enough cell surface VEGF receptors for that purpose. In contrast, endothelial cells recruited during angiogenesis express numerous VEGF receptors, but produce little or no detectable VEGF ligand. In this context, the amount of

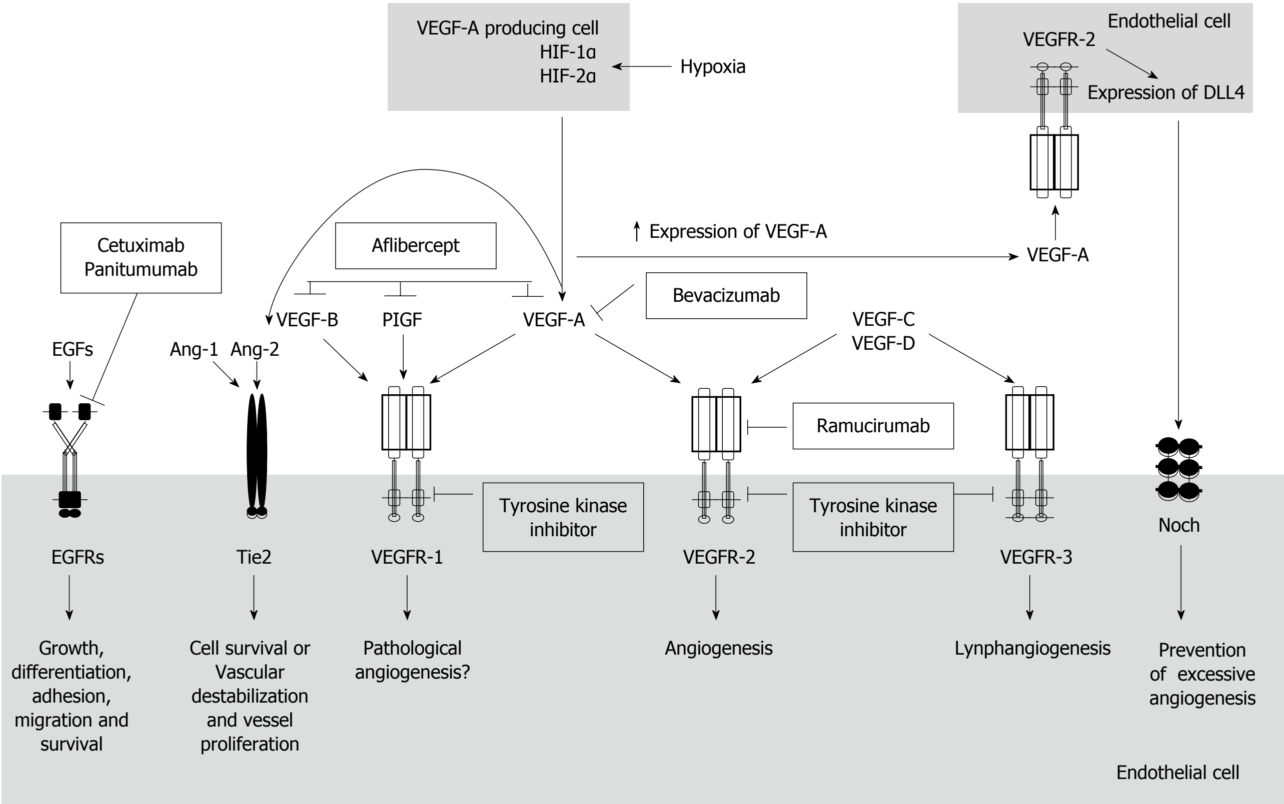

Figure 1 Main angiogenesis pathways involving vascular endothelial growth factor and the interactions with anti-angiogenic target drugs. VEGF: Vascular endothelial growth factor. HIF: Hypoxia-inducible factor; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; PIGF: Placental growth factor.

VEGF necessary to propel angiogenesis comes from several host cells in human body, such as platelets, smooth muscle cells, and tumor-associated stromal cells, which, together, produce the necessary amounts of VEGF for angiogenesis to begin[30-32].

The other VEGF family members are important for diverse mechanisms of new vessel creation. VEGF-B and PIGF bind to VEGFR-1 whereas VEGF-C and -D are specific ligands for VEGFR-2 VEGF receptor 3 (VEGFR-3), after proteolysis processing[26]. VEGF-B and PIGF act through VEGFR-1, which is capital for the organization of embryonic vasculature, but is not essential for endothelial cell differentiation[33,34]. VEGFR-1 is expressed in many non-endothelial cells such as monocyte/macrophages, dendritic cells, osteoclasts, pericytes and trophoblasts in the placenta. The value of VEGFR-1 expression in these cells remains unclear; however, this receptor could play a regulatory role in cell survival[26]. Furthermore, recent studies have shown that VEGFR-1 is present and functional on CRC cells and its activation, by VEGF family ligands, can result in activation of processes involved in tumor progression and metastasis[33]. VEGF-C and -D play an important role in lymphangiogenesis through VEGFR-2 and -3 binding[26]. Concerning VEGF-C, it is involved both in lymphangiogenesis and in promotion of metastasis to regional lymph nodes in multiple cancers, including CRC[35]. VEGF-D is also implicated in lymphangiogenesis and lymphatic metastasis[36].

Angiogenesis is regulated not only by VEGF pathways but also by other pathways including Notch, angiopoietins and integrins[20]. The Notch pathway, an intercellular signaling pathway, influences many biological processes, including cell fate determination, cellular differentiation, proliferation, survival and apoptosis[37,38]. There are four Notch cell-surface receptors (Notch-1, -2, -3 and -4) and five Notch membrane-anchored ligands [Jagged-1, Jagged-2, Delta-like (Dll)-1, -3, and -4], expressed by various cell types. Both ligand and receptor are transmembrane proteins with large extracellular domains that consist of epidermal growth factor (EGF)-like repeats. Notch is synthesized as a precursor protein that is processed in the Golgi before being transported to the cell surface, where it resides as a heterodimer. Interaction of Notch receptors with Notch ligands, between two bordering cells, initiates a series of successive proteolysis cleavages. The first cleavage, mediated by ADAM-family metalloproteases such as ADAM10 or tumor necrosis factor α-converting enzyme (TACE), generates a substrate for cleavage by the gamma-secretase complex. This cleavage leads to the release of Notch intracellular domain (NICD) from the cell membrane. This protein fragment, then, translocates into the nucleus and operates as a cofactor to regulate transcription of Notch target genes[39]. The induction of Dll4-Notch signaling acts as a mechanism intended to prevent excessive angiogenesis and to control the development of new blood vessels[40].

Vascular endothelial cells express Notch 1 and Notch

4 receptors and the Jagged-1, Dll1, and Dll4 ligands. Among these, Dll4 is expressed exclusively by endothelial cells[20]. Dll4 is usually induced by VEGF as a negative-feedback regulator of vascular growth. In contrast to VEGF blockade, which results in a loss of many tumor vessels and an apparent normalization of the remaining vessels of the tumor, DLL4 blockade results in a striking increase in these vessels. Paradoxically, this increased vascularity is associated with decreased tumor growth, even for tumors that are highly resistant to blockade of VEGF[41]. Since VEGF induces Dll4 and Dll4 induces vascular quiescence and differentiation, and down-regulates VEGFR-2[42], it is obvious that the balance of these two pathways may be important to the development and outcomes of therapeutic acting in these pathways[43].

Recently, the angiopoietins have emerged as important regulators of angiogenesis[16]. The human angiopoietin family comprises Ang-1, -2 and -3, all of which act as ligands for endothelial cell-specific tyrosine kinase receptor Tie2, expressed principally on the vascular endothelial cells[44-46].

Ang-1, which is predominantly expressed in perivascular cells such as pericytes, vascular smooth muscle cells, fibroblasts and tumor cells, binds to Tie2 receptor as an antagonist. Upon binding of Ang-1, Tie-2 receptor autophosphorylates, leading to stimulation of various intracellular signaling pathways which promote endothelial cell survival and the maintenance of an endothelial barrier and a quiescent vasculature. Mural cells, such as vascular smooth muscle cells and pericytes, constantly produce Ang-1 under physiological conditions, and maintain vascular stabilization and maturation[47]. On the other hand, Ang-2 produced by the endothelium, acts as an antagonist for Tie2 by competing with Ang-1[45]. It induces vascular destabilization and vessel proliferation. VEGF and angiopoietins have complementary roles in angiogenesis. In the presence of VEGF, Ang-2 stimulates tumor angiogenesis by promoting vessel destabilization, whereas in the absence of VEGF, Ang-2 promotes endothelial cell death and vessel regression[48].

Blockade of Tie-2 pathway has been more difficult than blockade of the VEGF pathway, due to the complexity of agonistic and antagonistic ligands for the same receptor. Moreover, it has been a challenge to find and design effective and specific drugs against Tie-2 or angiopoietins[20].

Fibroblast growth factor (FGF)/FGF receptor (FGFR) signaling is involved in multiple cellular processes, such as proliferation, anti-apoptosis, drug resistance, and angiogenesis[49]. FGFs are heparin-binding growth factors that are part of a family that comprises 23 members (FGF-1 to -23), of which only 18 are functional ligands for FGFR in humans. The members of the FGFR family (FGFR-1 to -4) share a common domain architecture consisting of extracellular immunoglobulin-like domains and a cytoplasmic tyrosine kinase domain[50]. Although FGF1 and FGF2 are among the first discovered molecules that contribute to angiogenesis, some members of the VEGF ligand family and VEGFR are now accepted to play a main role in driving embryonic vascularization, angiogenesis and lymphangiogenesis[51]. Nevertheless, both FGFs and VEGF cooperate to promote angiogenesis. FGF-2 induces the expression of VEGF in vascular endothelial cells, while the blockade of VEGF reduces the expression of endogenous FGF-2, suggesting a positive feedback mechanism. Furthermore, inhibition of FGFR-1 and FGFR-2 activity can reduce tumor vascularization as well as VEGF expression. Therefore, promotion of angiogenesis by FGFs may be dependent of crosstalk between FGF-VEGF signaling pathways[52].

EGF signaling is initiated by the binding of EGF family members to the extracellular domain of erythroblastic leukemia viral oncogene homologue (ErBb) receptors. The ErBb receptor tyrosine kinase family comprises 4 members, namely, EGF receptor (EGFR)/ERBB1/HER1, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4[53]. The major contributors of these receptors are a complex signaling cascade that modulates growth, signaling, differentiation, adhesion, migration and survival of cancer cells[54].

The EGF family members bind to the ErbB receptors and are classified into 3 groups based on their receptor affinities: in the first group, EGF, transforming growth factor-α, amphiregulin (AR), and epigen (EPG), specifically bind to EGFR; in the second group, betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), which exhibit dual specificity, bind to both EGFR and ErbB4; and the third group, which includes neuregulins (NRGs), forms two subgroups on the basis of their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4)[53,55]. On binding, ErbBs form homo or heterodimers and initiate multiple pathways involving effectors including rat sarcoma viral oncogene homologue (RAS)/mitogen-activated protein kinase, phosphatidylinositol 3-kinase-AKT, mammalian target of rapamycin, signal transducer and activator of transcription, SRC tyrosine kinase, phospholipase C-γ1/protein kinase C (PKC) and p27. The activation of these pathways plays a relevant role in several aspects of development and tissue homoeostasis[54]. Increased EGFR signaling is particularly common in several cancers, including CRC, through one or more of the family members[56]. EGFR and its family members, due to their vast role in the progression of cancer, have emerged as attractive candidates for anti-cancer therapy.

TREATMENT OF mCRC

Nowadays, there are many therapeutic strategies approved by the Food and Drug Administration (FDA) for the management of mCRC: 5-fluorouracil (5-FU), leucovorin (LV), irinotecan, capecitabine, oxaliplatin, regorafenib, ziv-aflibercept, and the monoclonal antibodies bevacizumab, cetuximab, and panitumumab. Of these drugs, only few have FDA-approved indications for use as monotherapies and reveal activity as a single agent

Table 1 Clinical trials and main anti-angiogenic drugs in metastatic disease

Clinical trial	Phase	Line	Regimen	Median PFS (mo)	Median OS (mo)	ORR (%)
Aflibercept						
VELOUR	Ⅲ	2nd	FOLFIRI + aflibercept *vs*	6.90 *vs* 4.67	13.50 *vs* 12.06	19.8 *vs* 11.1
NCT00561470[87]			FOLFIRI + placebo	HR = 0.758, P = 0.0001	HR = 0.817, P = 0.0032	P = 0.001
AFFIRM	Ⅱ	1st	mFOLFOX6 + aflibercept	8.48 *vs* 8.77		49.1 *vs* 45.9
NCT00851084[86]			*vs* mFOLFOX6			
Brivanib						
NCT00640471[90]	Ⅲ	3rd	Cetuximab + brivanib *vs*	5.0 *vs* 3.4	8.8 *vs* 8.1	13.6 *vs* 7.2
			cetuximab + placebo	HR = 0.72, P < 0.001	HR = 0.88, P = 0.12	P = 0.004
Regorafenib						
CORRECT	Ⅲ	2nd	Regorafenib *vs* placebo	1.9 *vs* 1.7	6.4 *vs* 5.0	
NCT01103323[99]				HR = 0.49, P < 0.000001	HR = 0.77, P = 0.0052	
Sorafenib						
RESPECT	Ⅱ	1st	mFOLFOX6 + sorafenib	9.1 *vs* 8.7	17.6 *vs* 18.1	
NCT00865709[107]			*vs* mFOLFOX6 + placebo	HR = 0.88, P = 0.46	HR = 1.13, P = 0.51	
Sunitinib						
NCT00668863	Ⅱ	1st				
NCT00457691[108]	Ⅲ	1st	FOLFIRI + sunitinib *vs*	7.8 *vs* 8.4	20.3 *vs* 19.8	32 *vs* 34
			FOLFIRI + placebo	HR = 1.095, P = 0.807	HR = 1.171, P = 0.916	P = 0.683
Valatanib						
CONFIRM1	Ⅲ	1st	FOLFOX4 + vatalanib *vs*	7.7 *vs* 7.6	21.4 *vs* 20.5	P > 0.05
NCT00056459[110]			FOLFOX4 + placebo	HR = 0.88, P = 0.118	HR = 1.08, P = 0.260	
CONFIRM 2	Ⅲ	2nd	FOLFOX4 + vatalanib *vs*	5.6 *vs* 4.2	13.1 *vs* 11.9	
NCT00056446[111]			FOLFOX4 + placebo	HR = 0.83, P = 0.013	HR = 1.00, P = 0.957	

mCRC: Metastatic colorectal cancer; PFS: Progression-free-survival; OS: Overall survival; ORR: Overall response rate; FOLFIRI: 5-fluorouacil + leucovorin + irinotecan; mFOLFOX6: 5-fluoroaucil + leucovorin + oxaliplatin; HR: Harzard ratio.

against CRC, including fluoropyrimidines (5-FU and capecitabine), irinotecan, cetuximab, and panitumumab.

The combination chemotherapy is the only standard for first-line treatment of mCRC. Regardless of which regimen is used, outcome may be maximized in patients who receive, alone or in combination, 5-FU, LV, irinotecan, and oxaliplatin sometime during the course of treatment. These chemotherapy regimens have been extensively studied in phase Ⅱ and Ⅲ trials, both as first- and second-line therapies[57,58]. Tables 1 and 2 summarizes current and future trials on mCRC anti-angiogenic therapies.

Antiangiogenic drugs

Bevacizumab is a humanized monoclonal antibody that binds and inactivates VEGF, preventing angiogenesis and, hence, tumor growth and proliferation. Bevacizumab contains human framework regions and the complementarity-determining regions of a murine antibody that inhibits all active isoforms of VEGF[59]. Currently, bevacizumab is the only agent specifically targeting the VEGF pathway for the treatment of CRC[60].

Over the past decades, many trials have investigated bevacizumab in mCRC. It has been studied with different active chemotherapy and biological agents, as well as in multiple treatment setting, sequencing and duration[61].

The phase Ⅱ trial conducted by Kabbinavar *et al*[62] compared two doses of bevacizumab plus 5-FU/LV (low-dose bevacizumab: 5 mg/kg every 2 wk; high-dose bevacizumab: 10 mg/kg every 2 wk) with 5-FU/LV alone in 104 patients untreated. Compared with the 5-FU/LV control arm, treatment with bevacizumab (at both dose levels) plus 5-FU/LV resulted in a higher response rate (RR) (control arm: 17%; low-dose arm: 40%; high-dose arm: 24%), longer median time to disease progression (control arm: 5.2 mo; low-dose arm: 9.0 mo; high-dose arm: 7.2 mo), and longer median survival (control arm: 13.8 mo; low-dose arm: 21.5 mo; high-dose arm: 16.1 mo). Based on these results, in the most subsequent phase Ⅲ trials on mCRC the 5 mg/kg bevacizumab dosing is chosen[61].

The phase Ⅲ AVF 2107 trial (NCT00109070) randomized 813 patients to receive IFL plus either bevacizumab 5 mg/kg (n = 402) or placebo (n = 411), every 2 wk. The addition of bevacizumab compared with IFL alone provided significantly clinical and statistical improvement in median OS (20.3 mo *vs* 15.6 mo; HR = 0.66, P < 0.001), PFS (10.6 mo *vs* 6.2 mo, HR = 0.54, P < 0.001) and overall response rate (ORR) (44.8% *vs* 34.8%, P = 0.004)[13].

In the NO16966 phase Ⅲ trial (NCT00069095), with 2 × 2 factorial design, 1401 patients with mCRC were randomized to receive FOLFOX or XELOX and then bevacizumab or placebo. Median PFS was significantly increased when bevacizumab was added (9.4 mo in bevacizumab group *vs* 8.0 mo in placebo group; HR = 0.83, P = 0.0023). Median OS was 21.3 mo in the bevacizumab group and 19.9 mo in the placebo group (HR = 0.89, P = 0.077), and RR was similar in both arms. A planned subset analysis demonstrated significant improvement of PFS with bevacizumab in the XELOX subgroup (P = 0.0026), as opposed when FOLFOX4 (P = 0.187) was added. Safety results showed that grade 3 or higher adverse events were slightly higher in the bevacizumab

Table 2 Current clinical trials considering anti-angiogenic therapies for colorectal cancer

	Trial	Phase	Line	Therapy/arms	Status of trial
Bevacizumab	NCT01321957	II	1st	FOLFOX + bevacizumab *vs* FOLFOX + bevacizumab + irinotecan	Currently recruiting participants
	NCT00819780	II	1st	Panitumumab + mFOLFOX6 *vs* bevacizumab + mFOLFOX6	Ongoing, but not recruiting participants
	NCT01531595	II	1st	3 cycles of XELOX + bevacizumab alternating with 3 cycles of XELIRI + bevacizumab	Currently recruiting participants
	NCT01067053	II	1st	Bevacizumab + capecitabine + oxaliplatin - 6 cycles; after the first 6 cycles of treatment, continuing only with bevacizumab and capecitabine	Ongoing, but not recruiting participants
	NCT01765582	II	1st	FOLFOXIRI + bevacizumab *vs* Sequential FOLFOXIRI + bevacizumab *vs* FOLFOX + bevacizumab	Currently recruiting participants
	NCT01006369	II	-	FOLFOX6 + bevacizumab + hydroxychloroquine *vs* XELOX + bevacizumab + hydroxychloroquine	Currently recruiting participants
	NCT01417494	II	1st	Chemotherapy (FOLFIRI, FOLFOX, LV5FU2) + bevacizumab *vs* Chemotherapy (FOLFIRI, FOLFOX, LV5FU2)	Currently recruiting participants
	NCT01532804	II	2nd	FOLFOX6 + bevacizumab (day 1 = day 15, 12 cycles) *vs* Raltitrexed + oxaliplatin + bevacizumab (day 1 = day 21, 8 cycles)	Currently recruiting participants
	NCT00952029	II / III	1st	FOLFIRI + bevacizumab and during the chemotherapy-free interval maintenance with bevacizumab *vs* FOLFIRI + bevacizumab and during the chemotherapy-free interval NO maintenance	Currently recruiting participants
Cetuximab	NCT01640405	III	1st	mFOLFOX6 + bevacizumab *vs* FOLFOXIRI + bevacizumab	Currently recruiting participants
	NCT00444678	II	-	Cetuximab + capecitabine + oxaliplatin	Ongoing, but not recruiting participants
	NCT01251536	II	1st	Cetuximab (standard dose: 250 mg/m^2 weekly) *vs* Cetuximab (dose escalation: days 22 and 29-350 mg/m^2, from day 36 onwards - 500 mg/m^2 weekly)	Currently recruiting participants
	NCT01718808	II	1st	Cetuximab + capecitabine *vs* Cetuximab	Currently recruiting participants
	NCT01867697	II	1st	Cetuximab (biweekly) + FOLFIRI (continuously) *vs* Cetuximab (biweekly) + alternating FOLFIRI and mFOLFOX6	Currently recruiting participants
	NCT00640081	II	1st	Intermittent chemotherapy plus intermittent cetuximab treatment (12 wk), plus cetuximab followed by a period off all therapy; reintroduction of the same chemotherapy and cetuximab regimen (12 wk after initial progression off treatment) *vs* Intermittent chemotherapy plus continuous cetuximab treatment (12 wk), plus cetuximab followed by a period of withdrawal of the chemotherapy, but continued weekly cetuximab monotherapy with reintroduction of the same chemotherapy regimen to the cetuximab (12 wk after initial progression off chemotherapy treatment)	Ongoing, but not recruiting participants
	NCT00479752	II	1st	FOLFOX4 + cetuximab (initial dose: 400 mg/m^2 in week 1, followed by weekly doses of 250 mg/m^2) *vs* FOLFOX4 + cetuximab (500 mg/m^2 every 2 wk)	Ongoing, but not recruiting participants
	NCT00482222	III	1st	Oxaliplatin/fluoropyrimidine *vs* oxaliplatin/fluoropyrimidine + cetuximab pre and post surgery	Currently recruiting participants
	NCT00433927	III	1st	FOLFIRI + cetuximab *vs* FOLFIRI + bevacizumab	Ongoing, but not recruiting participants
Panitumumab	NCT01228734	III	1st	Cetuximab + FOLFOX4 *vs* FOLFOX4	Ongoing, but not recruiting participants
	NCT01030042	III	2nd	FOLFOX4 followed, after progression, by irinotecan + cetuximab *vs* Cetuximab + irinotecan	Currently recruiting participants
	NCT00885885	II	-	Panitumumab + FOLFOX4 *vs* Panitumumab + FOLFOX4	Ongoing, but not recruiting participants
	NCT01215539	II	1st	Panitumumab + capecitabine + oxaliplatin	Currently recruiting participants
	NCT01126112	II	1st	Panitumumab (6 mg/kg every 2 wk)	Ongoing, but not recruiting participants
	NCT00819780	II	1st	Panitumumab + mFOLFOX 6 *vs* bevacizumab + mFOLFOX 6	Ongoing, but not recruiting participants
	NCT01328171	II	1st	FOLFOXIRI + panitumumab *vs* FOLFOXIRI	Currently recruiting participants
	NCT01508000	II	1st	mFOLFOX6 (6 cycles after and before surgery) + surgery *vs* mFOLFOX6 + bevacizumab (6 cycles after and before surgery) + surgery *vs* mFOLFOX6 + panitumumab (6 cycles after and before surgery) + surgery	Not yet open for participant recruitment
	NCT01814501	II	2nd	5-FU + irinotecan + panitumumab	Currently recruiting participants
	NCT00940316	II	2nd	Erlotinib + panitumumab + irinotecan (treatment repeats every 2 wk) *vs* Erlotinib + panitumumab (treatment repeats every 2 wk) *vs* Erlotinib + panitumumab	Currently recruiting participants
	NCT00364013	III	1st	FOLFOX + panitumumab *vs* FOLFOX	Ongoing, but not recruiting participants

	NCT01910610	Ⅲ	1st	FOLFIRI + cetuximab, followed by oxaliplatin-based chemotherapy + bevacizumab *vs* OPTIMOX + bevacizumab, followed by irinotecan-based chemotherapy + bevacizumab, followed by an anti-EGFR agent (cetuximab +/- irinotecan or panitumumab) with or without irinotecan	Not yet open for participant recruitment
Aflibercept	NCT01669720	Ⅱ	2nd	Aflibercept *iv* (4 mg/kg every 2 wk)	Currently recruiting participants
	NCT01652196	Ⅱ	1st	Aflibercept *iv* + mFOLFOX 6 *iv* (days 1 and 15; repeats every 28 d)	Currently recruiting participants
	NCT01802684	Ⅱ	1st	Induction therapy (sequence #1) Regimen: Aflibercept + mFOLFOX7 - 6 cycles (3 mo) Maintenance after induction (sequence #2) First phase (sequence #2A); Regimen: Aflibercept + fluoropyrimidine (simplifed LV5FU2 or capecitabine) - 6 cycles (3 mo) Second phase (sequence #2B); Regimen: Aflibercept +/- fluoropyrimidine (simplifed LV5FU2 or capecitabine) - until PD or limiting toxicity Reintroduction (sequence #3); Regimen: Aflibercept + mFOLFOX7 - 6 cycles (3 mo) Maintenance after reintroduction (sequence #4); Regimen: Aflibercept + fluoropyrimidine - until PD or limiting toxicity	Not yet open for participant recruitment
	NCT01882868	Ⅱ	2nd	Aflibercept *iv* + FOLFIRI Aflibercept + FOLFIRI (every 2 wk)	Currently recruiting participants
	NCT01889680	Ⅱ	1st	mFOLFOX6 + aflibercept (every 14 d for 6 cycles) plus 5-FU/LV (every 14 d) *vs* mFOLFOX6 + aflibercept (every 14 d for 6 cycles) plus 5-FU/LV + aflibercept (every 14 d)	Not yet open for participant recruitment
	NCT01646554	Ⅱ/Ⅲ	1st	mFOLFOX6 and SURGERY 6 cycles before and 6 cycles after surgery consisting in: Hour 0: Oxaliplatin 85 mg/m² *iv* 2-h infusion; Hour 0: Folinic acid 400 mg/m² (DL form) or 200 mg/m^2 (L form) *iv* 2-h infusion; Hour 2: 5-FU 400 mg/m² *iv* bolus over 2-4 min; Hour 2: 5-FU 2400 mg/m² given as a continuous infusion over 46 h; On day 1 of a 14 d cycle *vs* mFOLFOX6 + aflibercept and surgery; 6 cycles before and 6 cycles after surgery consisting in: Hour 0: Aflibercept 4 mg/kg intravenous infusion 1-h; Hour 1: Oxaliplatin 85 mg/m^2 2-h infusion; Hour 1: Folinic acid 400 mg/m^2 (DL form) or 200 mg/m^2 (L form) 2-h infusion; Hour 3: 5-FU bolus 400 mg/m^2 *iv* bolus over 2-4 min; Hour 3: 5-FU 2400 mg/m² given as a continuous infusion over 46 h; Day 1 of a 14 day cycle	Not yet open for participant recruitment
	NCT01661270	Ⅲ	2nd	Aflibercept *iv* (day 1 of each cycle, every 2 wk) + FOLFIRI *vs* Placebo *iv* (day 1 of each cycle, every 2 wk) + FOLFIRI	Currently recruiting participants
	NCT01571284	Ⅲ	2nd	Aflibercept IV (every 2 wk) + FOLFIRI	Currently recruiting participants
	NCT01670721	Ⅲ	2nd	Aflibercept IV (on day 1) + FOLFIRI administered as follows: dI-leucovorin infusion over 2 h on day 1; Irinotecan: infusion over 90-min infusion, on day 1, followed by bolus 5-FU and 5-FU continuous infusion over 46 h or as individualized by physician's clinical judgment; Treatment cycle to be administered every 2 wk	Currently recruiting participants
Brivanib	NCT01367275	Ⅱ	2nd	Brivanib (800 mg orally daily days 1-14) + Irinotecan *iv* (180 mg/m^2 on day 1)	Ongoing, but not recruiting participants
Cediranib	NCT00588900	Ⅱ	2nd	Irinotecan *iv* (days 1 and 8) + Cediranib oral (days 1-21)	The recruitment status of this study is unknown because the information has not been verified recently
Ramucirumab	NCT01111604	Ⅱ	2nd	mFOLFOX-6 *vs* mFOLFOX-6 + ramucirumab (8 mg/kg *iv* infusion, administered every 2 wk) *vs* mFOLFOX-6 + icrucumab (15 mg/kg *iv* infusion, administered every 2 wk)	Ongoing, but not recruiting participants
	NCT01183780	Ⅲ	2nd	FOLFIRI + ramucirumab (8 mg/kg administered intravenously every 2 wk) *vs* FOLFIRI + placebo	Currently recruiting participants
Regorafenib	NCT01298570	Ⅱ	2nd	Regorafenib (160 mg, *po*, daily, per 7 day cycle) + FOLFIRI (day 1 and day 15 of each 28 d cycle) *vs* Placebo (oral administration, days 4-10 and days 18-24 of 28 day cycle +)+ FOLFIRI (day 1 and day 15 of each 28 d cycle)	Currently recruiting participants
	NCT01289821	Ⅱ	1st	Day 1 and day 15 of each cycle: 85 mg/m² oxaliplatin + folinic acid (either 400 mg/m² D/L-folinic acid or 200 mg/m² L-folinic acid), *iv* + 400 mg/m² 5 FU *iv* + 2400 mg/m² 5 *iv*; Days 4 to 10 and days 18 to 24: regorafenib 160 mg (four 40 mg tablets)	Ongoing, but not recruiting participants
	NCT01875380	Ⅱ	1st	Regorafenib (orally, 160 mg per day for 3 wk, followed by 1 wk of rest)	Not yet open for participant recruitment
	NCT01103323	Ⅲ	2nd	Regorafenib (160 mg per oral once daily for 3 wk on 1 wk off of every 4 wk cycle) *vs* Placebo (per oral once daily for 3 wk on 1 wk off of every 4 wk cycle)	Ongoing, but not recruiting participants
	NCT01584830	Ⅲ	2nd	Regorafenib [3 wk on/1 wk off (160 mg od *po*)] Placebo [3 wk on/1 wk off (160 mg od *po*)]	Ongoing, but not recruiting participants
	NCT01853319	Ⅲ	2nd	Regorafenib (160 mg per oral every day for 3 wk of every 4 wk cycle)	Not yet open for participant recruitment
	NCT01538680	Ⅲ	2nd	Regorafenib (160 mg *po* every day for 3 wk on, 1 wk off)	Expanded access is currently available for this treatment

Semaxanib	NCT00021281	Ⅲ	1st	Semaxanib *iv* (on days 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36 and 39) + irinotecan *iv*, leucovorin calcium *iv*, fluorouracil *iv* (on days 1, 8, 15, and 22) (every 6 wk) *vs* Irinotecan iv, leucovorin calcium iv, fluorouracil *iv* (on days 1, 8, 15, and 22) (every 6 wk) *vs* Semaxanib *iv* (on days 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36 and 39) + irinotecan *iv* (days 1, 15 and 29) + leucovorin calcium *iv*, fluorouracil *iv* (on days 1, 2, 15, 16, 29 and 30) *vs* Irinotecan *iv*, leucovorin calcium *iv*, fluorouracil *iv* (on days 1, 8, 15, and 22) (every 6 wk)	The recruitment status of this study is unknown because the information has not been verified recently
Sorafenib	NCT01715441 NEXIRI 2	Ⅱ	2nd	Irinotecan 180 mg/m^2 *iv* with cross over to irinotecan and sorafenib combination at progression *vs* Sorafenib 400 mg twice daily with cross over to irinotecan and sorafenib combination at progression *vs* Irinotecan 120 mg/m^2 *iv* at cycle 1, 150 mg/m² at cycle 2 and 180 mg/m² at cycle 3 + sorafenib 400 mg twice daily from cycle 1	Currently recruiting participants
	NCT01471353	Ⅱ	2nd	Sorafenib 200-400 mg *po* twice daily on days 1-21 (dose escalation scheme) + capecitabine 1000 mg/m^2 *po* twice daily on days 1-14 repeated every 21 d	Currently recruiting participants
	NCT00826540	Ⅱ	2nd	Sorafenib twice daily on days 1-5 and 8-12 + bevacizumab *iv* on day 1	Ongoing, but not recruiting participants
	NCT00839111	Ⅱ	2nd	Sorafenib (400 mg twice daily from day 3 to day 14, day 17-28) + FOLFIRI	The recruitment status of this study is unknown because the information has not been verified recently
	NCT01290926	Ⅱ	2nd	Sorafenib (200 mg in the morning, 400 mg in the evening) + capecitabine (850 mg/m^2 twice daily)	The recruitment status of this study is unknown because the information has not been verified recently
	NCT00326495	Ⅱ	2nd	Oral sorafenib (400 mg by twice daily) plus cetuximab (400 mg/m^2, week 1; 250 mg/m^2 *iv*, weekly)	Currently recruiting participants
Sunitinib	NCT00936832	Ⅱ	1st	FOLFIRI (on days 1, 15, and 29) + oral sunitinib (on days 1-28). (repeats every 6 wk)	The recruitment status of this study is unknown because the information has not been verified recently

Research on July 25, 2013 (http://clinicaltrials.gov.). 5-FU: 5-fluoraucil; FOLFIRI: 5-fluorouacil + leucovorin + irinotecan; mFOLFOX6: 5-fluoroaucil + leucovorin + oxaliplatin; HR: Harzard ratio.

group (30% *vs* 21%)[63].

In the phase Ⅲ MAX study, 471 patients with previously untreated and unresectable mCRC were randomly assigned to the following arms: capecitabine alone, capecitabine plus bevacizumab, or capecitabine, bevacizumab, and mitomycin. Median PFS was 5.7 mo for the capecitabine arm, 8.5 mo for the capecitabine-bevacizumab arm, and 8.4 mo for the capecitabine-bevacizumab-mitomycin arm. Thus, there was statistical improvement in PFS between the capecitabine arm and the other two arms (capecitabine *vs* capecitabine-bevacizumab: HR = 0.63, $P < 0.001$; capecitabine *vs* capecitabine-bevacizumab-mitomycin: HR = 0.59, $P < 0.001$)[64]. Based on these results, in United States and Europe, bevacizumab in association with standard chemotherapy has been approved for first-line treatment of *KRAS*-mutant mCRC or for second-line treatment of *KRAS*-wild type patients previously treated with anti-EGFR drugs.

Despite those interesting benefits reported in previous trials, researchers and clinicians should be knowledgeable about toxicities, such as for hypertension and bleeding.

Anti-EGFR agents

Cetuximab and panitumumab are two EGFR inhibitors currently indicated as monotherapy in patients with wild-type *KRAS* tumors as a first or second-line treatment[65]. Only cetuximab is indicated in combination with irinotecan, and has been approved for use in first-line in Europe as mono-therapy or in combination with chemotherapy[66].

Cetuximab is a recombinant human-murine chimeric IgG1 monoclonal antibody that binds to the extracellular region of the EGFR with high specificity and with higher affinity than EGF on normal and tumor cells[67].

A phase Ⅱ clinical trial conducted by Tabernero *et al*[68] assessed 43 patients who received cetuximab and FOLFOX4 as first-line chemotherapy. RR was 72%; median PFS was 12.3 mo and median OS was 30 mo. Cetuximab did not increase the characteristic toxicity of FOLFOX4 and was collectively well tolerated. The most commonly reported grade 3 or higher adverse events were diarrhea, neutropenia, and paresthesia.

The OPUS study, also a phase Ⅱ trial (NCT00125034), included 337 patients who were randomized to receive FOLFOX4 with cetuximab (n = 169) or alone (n = 168) in first-line chemotherapy[69]. In 93% of measured *KRAS* patient samples, 57% were *KRAS*-wild type. Patients whose tumors were *KRAS*-wild type who received cetuximab plus FOLFOX4 had a 2.6-fold increased odds ratio of response (ORR: 57% *vs* 34%, OR = 2.551, $P = 0.0027$) and a 43% decrease in the risk of disease progression (median PFS 8.3 mo *vs* 7.2 mo, HR = 0.567, $P = 0.0064$) compared with those who received FOLFOX4 alone. Also, median OS was improved by the addition of cetuximab to FOLFOX4 for patients in that group (22.8 mo *vs* 18.5 mo, HR = 0.855, $P = 0.39$). On the other hand, patients whose tumors carried KRAS mutations who

received cetuximab plus FOLFOX4 had a decreased odd of response (34% *vs* 53%, OR = 0.459, *P* = 0.0290) and a higher risk of disease progression (median PFS 5.5 mo *vs* 8.6 mo, HR = 1.720, *P* = 0.0153) compared with those who received FOLFOX4 alone[70].

In the phase Ⅲ CRYSTAL study (NCT00154102), 1198 patients who received cetuximab plus FOLFIRI (*n* = 599) or FOLFIRI alone (*n* = 599) were included. The addition of cetuximab to chemotherapy significantly reduced the risk of progression by 15% (8.9 mo *vs* 8.0 mo, HR = 0.85, *P* = 0.048) and improved ORR (46.9% *vs* 38.7%, OR = 1.40, *P* = 0.048). On the other hand, no significant difference in median OS between the two treatment groups was observed (19.9 mo *vs* 18.6 mo, HR = 0.93, *P* = 0.31)[71]. In that study, *KRAS* and *BRAF* mutations were detected in 37% and 6% of patients, respectively. The addition of cetuximab to FOLFIRI in patients with wild-type *KRAS* resulted in significant improvement in median OS (23.5 mo *vs* 20.0 mo, HR = 0.796, *P* = 0.0093), median PSF (9.9 mo *vs* 8.4 mo, HR = 0.696, *P* =0.0012), and RR (57.3% *vs* 39.7%, OR = 2.069, *P* < 0.001) compared with FOLFIRI alone. These results showed the role of KRAS mutation status as a powerful predictive biomarker for the efficacy of cetuximab plus FOLFIRI. Concerning grade 3 or 4 adverse events, they were more common with use of regimen with cetuximab and included skin reactions, infusion reactions and diarrhea[72].

In the phase Ⅲ study NORDIC VⅡ (NCT00145314), 571 patients with mCRC were randomized to one of the following three arms: continuous FLOX alone or with cetuximab or intermittent FLOX with continuous weekly cetuximab. No differences were found in RR, median PFS or OS in patients receiving cetuximab, either in *KRAS*-mutant or -wild-type[73].

In the phase Ⅲ trial MRC COIN, 1630 patients were randomized to receive oxaliplatin-based chemotherapy (FOLFOX or XELOX) with or without cetuximab. The determination of *KRAS* mutation was performed in 1316 (81%) patients and it was identified in 729 (55%) patients[74]. Patients with wild-type *KRAS* tumors showed no improvements in median OS for cetuximab combined with chemotherapy when compared with chemotherapy alone (17.0 mo *vs* 17.9 mo, HR = 1.038, *P* = 0.68) or PFS (8.6 mo *vs* 8.6 mo, HR = 0.96, *P* = 0.60); however, there was an increase in ORR (57% *vs* 64%, *P* = 0.049). Furthermore, there was a potential benefit with improvement in PFS for wild-type *KRAS* patients who received cetuximab plus infused 5-FU (HR = 0.72, *P* = 0.037) but not cetuximab plus capecitabine (HR = 1.02, 95%CI: 0.82-1.26, *P* = 0.88)[74].

Based on those trials, cetuximab in addition with standard chemotherapy has been approved in United States and Europe for wild-type *KRAS* mCRC patients in first-line regimen. It is important to monitor toxicity profile such as skin rash, diarrhea, nausea and mucositis in order to provide a good tolerability for patients. Regular medical visits before each cycle and support medication could help address this concern.

Panitumumab is a recombinant human IgG2κ monoclonal antibody that binds EGFR and prevents receptor dimerization, tyrosine autophosphorylation of EGFR, and the activation of downstream signaling molecules[75].

The phase Ⅲ trial PRIME (NCT00364013) included 1183 patients without prior chemotherapy for mCRC, who were randomly assigned to receive FOLFOX4 with or without panitumumab therapy. In the wild-type *KRAS* subgroup, panitumumab plus FOLFOX4 produced a significantly improved median PFS compared with FOLFOX4 alone (9.6 mo *vs* 8.0 mo, respectively; HR = 0.80, *P* = 0.02). Nevertheless, a non-significant increase in median OS was found for panitumumab plus FOLFOX4 versus FOLFOX4 alone (23.9 mo *vs* 19.7 mo, respectively, HR = 0.83, *P* = 0.072). In the mutant *KRAS* subgroup PFS was significantly reduced in the panitumumab plus FOLFOX4 arm when compared with the FOLFOX4 alone arm (HR = 1.29, *P* = 0.02), and median OS was 15.5 mo *vs* 19.3 mo, respectively (HR = 1.24, *P* = 0.068)[76].

As a conclusion, the use of cetuximab or panitumumab for wild-type *KRAS* mCRC patients will depend on the patient fitness, toxicity profile and drug wiliness in each circumstance. Both drugs are safe and prove to improve OS in the metastatic setting.

Double monoclonal antibody therapy

The efficacy of bevacizumab and anti-EGFR agents in first-line treatment of mCRC encouraged two clinical trials of double monoclonal antibody therapy[77].

In the phase Ⅲ PACCE (NCT00115765) study, a total of 1053 patients were randomized to receive first-line chemotherapy [oxaliplatin/5-FU/LV (*n* = 823 patients) or irinotecan/5-FU/LV (*n* = 230 patients)] and bevacizumab with or without panitumumab. The study was discontinued early after a planned interim analysis showed reduced PFS and increased toxicity in the panitumumab arm. In the final analysis, median PFS (10.0 mo *vs* 11.4 mo for the panitumumab and control arms, respectively, HR = 1.27) and OS (19.4 mo *vs* 24.5 mo for the panitumumab and control arms, respectively) were shorter in the panitumumab arm in the entire study cohort as well as in the subset with wild-type KRAS. Grade 3/4 adverse events in the oxaliplatin (panitumumab *vs* control) cohort included skin toxicity (36% *vs* 1%), diarrhea (24% *vs* 13%), infections (19% *vs* 10%), and pulmonary embolism (6% *vs* 4%)[78].

Similarly, in the phase Ⅲ CAIRO2 trial, 755 patients with previously untreated mCRC were randomly assigned to receive capecitabine, oxaliplatin, and bevacizumab (CB regimen, *n* = 378 patients) or the same regimen plus weekly cetuximab (CBC regimen, *n* = 377 patients). The addition of cetuximab to XELOX plus bevacizumab resulted in shorter PFS in the entire study cohort (10.7 mo in the CB group *vs* 9.4 mo in the CBC group, *P* = 0.01) and in the wild-type *KRAS* subset compared with XELOX plus bevacizumab. No difference in OS (20.3 mo in the CB group *vs* 19.4 mo in the CBC group, *P* = 0.16)

or ORR (50.0% in the CB group *vs* 52.7% in the CBC group, P = 0.49) was verified between treatment arms. Patients treated with cetuximab who had tumors bearing a mutated *KRAS* gene had significantly decreased PFS as compared with cetuximab-treated patients with wild-type KRAS tumors (8.1 mo *vs* 10.5 mo, P = 0.04) or patients with mutated KRAS tumors in the CB group (8.1 mo *vs* 12.5 mo, P = 0.003). Grade 3 or 4 adverse events were more frequent in the CBC group, which were attributed to cetuximab-related adverse cutaneous effects[79].

On the basis of these studies, double monoclonal antibody therapy with bevacizumab and an anti-EGFR agent is not recommended[77].

Management of liver metastasis

In order to determine the treatment strategy for hepatic metastases of CRC, it is important to verify the presence of one of three situations: metastases are readily resectable; metastatic disease is initially considered to be unresectable, principally due to location; or liver metastases are unlikely ever to become resectable[80]. Surgical resection undoubtedly remains the gold standard for the treatment of resectable colorectal liver metastases because it improves patient's prognosis if the metastases are resectable. When surgery is not indicated for hepatic metastases, chemotherapy is the first-choice treatment. In cases where surgical resection becomes possible and chemotherapy is effective, the long-term prognosis may be good[81].

For patients with initially resectable disease, with good prognostic factors, one approach is immediate surgical resection and another is perioperative chemotherapy such as FOLFOX4[82,83]. Today, chemotherapy before surgery, even in patients with resectable metastases, can increase the complete resection rate, facilitate limited hepatectomies, improve postoperative recovery, treat micrometastases, provide a test of chemoresponsiveness, identify aggressive disease, spare ineffective therapy and prolong relapse-free survival[80].

In potentially resectable colorectal liver metastases, neoadjuvant chemotherapy, infused 5-FU/LV, in combination with either irinotecan or oxaliplatin, as well as triple cytotoxic drug therapy, *e.g.*, FOLFOXIRI, and more recent combination chemotherapy regimens with targeted agents cetuximab and bevacizumab, should be considered to enhance the chance of cure of patient with initially unresectable liver metastases[80,82].

In liver metastases that are unlikely to ever become resectable, palliative chemotherapy based on FOLFOX4/XELOX, FOLFIRI, with or without biological therapies, should be considered. In this setting, the possibility of doing a resection should not be excluded[82].

TARGET THERAPIES-OTHERS

Others drugs are also under investigation or have been recently approved for the use in the metastatic setting, as showed in Tables 1 and 2. Further down, we will discuss the main trials in each field.

Aflibercept

Aflibercept (Ziv-aflibercept, VEGF-Trap) is a recombinant VEGFR-antibody protein generated by the fusion of second immunoglobulin (Ig) domain of the VEGFR-1 and the third Ig domain of the VEGFR2 to the Fc domain of human IgG1[84]. In contrast to bevacizumab, which only binds to VEGF-A and forms multimeric complexes, aflibercept traps the different isoforms of VEGF-A, with approximately 1000-fold higher affinity than bevacizumab. In addition, aflibercept binds to VEGF-B and PIGF[85]. This VEGF-Trap effectively suppresses tumor growth and vascularization *in vivo*, resulting in stunted and almost completely avascular tumors[84].

To investigate the potential role of aflibercept in the first-line treatment of mCRC with chemotherapy, the phase Ⅱ AFFIRM trial (NCT00851084) recruited 236 patients who had never received therapy for mCRC or angiogenesis inhibitors. A total of 117 patients received mFOLFOX6 alone and 119 received mFOLFOX6 plus aflibercept (4 mg/kg *iv* every 2 wk). This study showed similar efficacy of FOLFOX plus aflibercept *vs* FOLFOX alone with respect to ORR (49.1% *vs* 45.9%, respectively) and median PFS (8.48 mo *vs* 8.77 mo, respectively)[86].

The purpose of the phase Ⅲ randomized, placebo-controlled clinical trial VELOUR (NCT00561470) was to investigate the efficacy and safety of aflibercept plus FOLFIRI in the second-line treatment of mCRC after oxaliplatin failure. 614 participants were randomly assigned to receive aflibercept (4 mg/kg intravenously; 612 patients) or placebo (614 patients) every 2 wk in combination with FOLFIRI. Median OS was 13.50 mo for aflibercept and 12.06 mo for placebo (HR = 0.817, P = 0.0032). Adding aflibercept to FOLFIRI also increased PFS relative to placebo plus FOLFIRI (HR = 0.758, P = 0.0001), with median PFS times of 6.90 mo *vs* 4.67 mo, respectively. The ORR in the aflibercept group was 19.8% compared with 11.1% in the placebo group (P = 0.001). Grade 3/4 adverse events with an at least 2% higher incidence with aflibercept versus placebo were diarrhea, asthenia/fatigue, stomatitis/ulceration, infections, hypertension, gastrointestinal/abdominal pain, neutropenia/neutropenic complications and proteinuria[87]. Approximately one third of study participants had previously been treated with bevacizumab (187 in the placebo and 186 in the aflibercept group). Aflibercept produced a consistent trend towards prolonged OS (P = 0.7231) and PFS (P = 0.6954), regardless of prior use of bevacizumab. The incidence of adverse events in the aflibercept arm was similar in patients with prior bevacizumab (100%) to those without (98.9%), with a similar incidence of grade 3/4 events (82.5% and 83.9%, respectively). Results of this subgroup analysis showed that the addition of aflibercept to FOLFIRI leads to a consistent trend of increased OS and PFS, regardless of prior bevacizumab use[88].

Brivanib

Brivanib alaninate (BMS582664) is an oral, potent selective inhibitor of both the FGF and VEGF family of receptors[89]. Besides its antiangiogenic activity from blocking VEGFR-2 and -3, its ability to disrupt FGF receptors (FGFRs) -1, -2 and -3 has been suggested to circumvent primary and/or acquired resistance to VEGF blockade, and block FGF-dependent tumor proliferation[90]. In preclinical studies using *in vivo* tumor xenograft models of CRC resistant to bevacizumab, the strong antiangiogenic effects and antitumor activity of brivanib[91] were established. Phase Ⅰ studies evaluated brivanib in combination with cetuximab in advanced gastrointestinal malignancies, including CRC, and demonstrated good tolerability and some evidence of clinical activity[92,93].

A phase Ⅲ study (NCT00640471) was carried out to evaluate combined use of brivanib and cetuximab without chemotherapy in third-line therapy for mCRC. A total of 750 patients were randomly assigned to treatment: 376 on brivanib plus cetuximab arm and 374 on placebo plus cetuximab arm. Patients included in this trial had wild-type *K-RAS*, had received prior fluoropyrimidine, and had been treated with irinotecan and oxaliplatin. Despite positive effects on PFS (5.0 mo in brivanib arm and 3.4 mo in placebo arm-HR = 0.72, $P < 0.001$) and objective response, cetuximab plus brivanib increased toxicity and did not significantly improve OS in patients with metastatic, chemotherapy-refractory, wild-type *K-RAS* colorectal cancer (8.8 mo in brivanib arm and 8.1 mo in placebo arm-HR = 0.88, $P = 0.12$). A total of 51 patients in brivanib arm and 27 patients in placebo arm had complete or partial response, yielding ORR of 13.6% and 7.2% for brivanib and placebo arms, respectively. The difference in ORR was statistically significant, supporting the brivanib plus cetuximab combination ($P = 0.004$). The median duration of response was 5.8 mo in brivanib arm and 5.4 mo in placebo arm ($P = 0.04$). Incidence of grade 3 or higher adverse events was 78% in brivanib arm and 53% in placebo arm, particularly fatigue, hypertension, rash, abdominal pain, diarrhea, dehydration, and anorexia. Hematologic adverse events were uncommon in both arms[90].

Cediranib

Cediranib (AZD2171) is a highly potent and selective inhibitor of the three VEGFRs and has a half-life suitable for once-daily oral dosing[94]. Cediranib is currently in phase Ⅲ development for the first-line treatment of mCRC. The clinical development program includes two global phase Ⅱ/Ⅲ studies (HORIZON Ⅱ and HORIZON Ⅲ) in the first-line treatment setting, and a phase Ⅱ study in second-line treatment.

HORIZON Ⅱ (NCT00399035) is a randomized phase Ⅱ/Ⅲ trial aimed to compare chemotherapy (FOLFOX or XELOX) with cediranib or placebo as first-line therapy in patients with mCRC. In this study, cediranib plus chemotherapy significantly improved PFS (HR = 0.84) but not OS (HR = 0.94) or ORR, compared with placebo plus chemotherapy.

HORIZON Ⅲ (NCT00384176) incorporated a phase Ⅱ/Ⅲ study design. An end-of-phase-Ⅱ analysis of efficacy and safety was undertaken to determine whether the study should continue into the phase Ⅲ part. In this study, a randomized comparison of mFOLFOX6 in combination with cediranib versus mFOLFOX6 in combination with bevacizumab as first-line chemotherapy was made in patients with mCRC.

Ramucirumab

Ramucirumab (IMC-1121B) is a fully humanized IgG1 monoclonal antibody that binds with high affinity to the extracellular VEGF-binding domain of VEGFR-2. Ramucirumab binds to a VEGFR-2 epitope involved in ligand binding and blocks VEGF ligands from binding this site and activating the receptor[95]. The inhibition of VEGF-stimulated VEGFR-2 activation provides ramucirumab significant antitumor activity in a range of malignancies in animal models as a single agent or in combination with other therapeutics[96].

Several studies assessing ramucirumab in mCRC are currently underway (Table 2), without reported results. In a phase Ⅱ study (NCT00862784) participants were treated with ramucirumab (8 mg/kg infusions every 2 wk) in combination with mFOLFOX6 as first-line therapy. In another phase Ⅱ study (NCT01111604), patients with disease progression on an irinotecan-based, first-line chemotherapy regimen (FOLFIRI or CAPIRI) received mFOLFOX-6 alone or in combination with ramucirumab (8 mg/kg infusions every 2 wk). The phase Ⅱ study NCT01079780 evaluated the combination of ramucirumab, cetuximab, and irinotecan versus cetuximab and irinotecan in patients with mCRC and progression following a bevacizumab-based regimen. A phase Ⅲ study (NCT01183780) evaluates the role of ramucirumab, in combination with FOLFIRI chemotherapy, in patients with progression following first-line combination therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine. Soon, ramucirumab may show its place in the current clinical practice scenario.

Regorafenib

Regorafenib (BAY 73-4506) is an oral multikinase inhibitor that blocks the activity of multiple protein kinases, including kinases involved in the regulation of tumor angiogenesis (VEGFR-1, -2, and -3, and angiopoietin-1 receptor), oncogenesis (KIT, RET, RAF1, BRAF, and BRAFV600E), and the tumor microenvironment (PDGFR and FGFR)[97]. Preclinical studies (both *in vitro* and *in vivo*) showed a broad spectrum of antitumor activity of Regorafenib as a result of its ability to block several angiogenic, stromal and oncogenic kinases[98].

The phase Ⅲ trial CORRECT (NCT01103323) investigated the use of regorafenib in 760 patients who had received all locally approved standard therapies and had progressed during or within 3 mo after the last standard therapy. Patients were randomized in a 2:1 ratio to

receive regorafenib (160 mg orally daily for 3 out of 4 k; n = 500) versus placebo (3 wk on and 1 wk off; n = 253), respectively. Randomization was based on pre-allocated block sizes and patients were stratified by previous treatment with VEGF-targeting drugs, time from diagnosis of metastatic disease (⩾ 18 or < 18 mo), and geographical region. This study reported an increase in OS for regorafenib-treated patients against best supportive care, after progression on standard therapy (6.4 mo *vs* 5.0 mo, respectively, HR = 0.77, one-sided P = 0.0052). Also, median PFS was 1.9 mo *vs* 1.7 mo when compared with placebo (HR = 0.49, one-sided P < 0.000001). After the interim analysis, the study was unblinded and patients were allowed to cross over to the regorafenib arm. Treatment-related adverse events occurred in 93%of patients in the regorafenib arm and in 61% of those in the placebo arm. The most common grade 3 or higher side effects related to regorafenib were hand-foot skin reaction (17%), fatigue (10%), diarrhoea (7%), hypertension (7%), and rash or desquamation (6%)[99]. Based on the CORRECT study, regorafenib received approval from the FDA in October 2012 for the treatment of chemorefractory mCRC patients. However, we believe that this drug should be provided only in a specific context due to the modest results reported on OS benefit and pharmaco-economic evaluation.

Semaxanib

Semaxanib (SU5416) is a potent, specific and competitive (with respect to ATP) inhibitor of the tyrosine kinase activity of Flk-1/KDR. Semaxanib was shown to inhibit VEGF-dependent mitogenesis of human endothelial cells, without inhibiting the growth of a variety of tumor cells *in vitro*[100,101].

A clinical phase Ⅲ study (NCT00004252) studied the combination of 5-FU/LV with semaxanib or alone, as a first-line therapy for mCRC patients. Although the study had already been completed, its results are not yet known.

Sorafenib

Sorafenib is an oral multikinase inhibitor with anti-proliferative and anti-angiogenic effects. It inhibits the activity of the serine/threonine kinases c-Raf and B-Raf; the mitogen-activated protein kinases MEK and ERK; VEG; PDGFR; the cytokine receptor c-KIT; the receptor tyrosine kinases Flt-3 and RET; and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway[102]. *In vivo* and *in vitro* studies showed that sorafenib inhibits tumor growth and disrupts tumor microvasculature through antiproliferative, antiangiogenic, and/or proapoptotic effects[103].

In the double-blind placebo-controlled phase Ⅱ study RESPECT (NCT00865709), the addiction of sorafenib to mFOLFOX6 was evaluated. 198 patients were randomized to receive sorafenib (400 mg *bid*) (n = 97) or placebo (n = 101), combined with mFOLFOX6 every 14 d. Median PFS was 9.1 mo for the sorafenib arm and 8.7 mo for the placebo arm (HR = 0.88, P = 0.46). Similar results were observed in the subgroup analyses: in patients with wild-type *KRAS*, the median PFS was 9.5 mo *vs* 9.2 mo, respectively (HR = 0.84), with corresponding medians of 7.8 mo *vs* 7.6 mo, respectively, in the mutant *KRAS* subgroup (HR = 0.96). In patients with wild-type *BRAF*, the median PFS was 9.2 mo *vs* 9.0 mo, respectively (HR = 0.91), and the median PFS for patients with mutant *BRAF* was 8.6 mo *vs* 7.3 mo, respectively (HR = 0.89). There was no difference between treatment arms for median OS (17.6 mo in the sorafenib arm *vs* 18.1 mo in the placebo arm-HR = 1.13, P = 0.51). In patients with wild-type *KRAS*, median overall survival was 19.9 mo *vs* 16.8 mo, respectively (HR = 0.89), and 17.0 mo *vs* 19.4 mo, respectively, in patients with mutant *KRAS* (HR = 1.29). In patients with wild-type *BRAF*, median overall survival was 18.8 mo *vs* 18.3 mo, respectively (HR = 1.09), and 13.9 mo *vs* 11.9 mo, respectively, in patients with mutant *BRAF* (HR = 0.46). The most common grade 3/4 adverse events in the sorafenib and placebo arms were neutropenia (48% *vs* 22%), peripheral neuropathy (16% *vs* 21%), and grade 3 hand-foot skin reaction (20% *vs* 0%). Treatment discontinuation because of adverse events was 9% and 6%, respectively. Generally, dose intensity (duration and cumulative doses) was lower in the sorafenib arm than in the placebo arm. This study did not detect a PFS benefit with the addition of sorafenib to first-line FOLFOX6 for mCRC, and *KRAS* and *BRAF* status did not seem to impact treatment outcomes. These results do not support further development of sorafenib in combination with mFOLFOX6 in molecularly unselected patients with mCRC[104].

The clinical phase Ⅱ study FOSCO (NCT00889343) studied the combination of FOLFOX6 or FOLFIRI with sorafenib or alone, as a second-line therapy in mCRC patients. Although the study had already been completed, its results are not yet known.

Sunitinib

Sunitinib malate (SUTENT) is an oral, multitargeted tyrosine kinase inhibitor that selectively inhibits the VEGFR and PDGFR family members, as well as stem-cell factor receptor (KIT), glial cell line-derived neurotrophic factor receptor (rearranged during transfection; RET), colonystimulating factor receptor (CSF-1R), and FMS-like tyrosine kinase-3[105-107].

In a phase Ⅲ trial (NCT00457691), 768 patients with mCRC were randomly assigned to receive intravenous FOLFIRI (every 2 wk) plus sunitinib (37.5 mg/d, 4 wk on, 2 wk off) (n = 386) or placebo (n = 382). Median PFS was 7.8 mo in the sunitinib plus FOLFIRI arm and 8.4 mo in the placebo plus FOLFIRI arm (HR = 1.095; P = 0.807), indicating a lack of superiority for sunitinib plus FOLFIRI. Median OS was 20.3 mo in the sunitinib arm and 19.8 mo in the placebo arm (HR = 1.171, one-sided stratified Log-rank P = 0.916). In addition, the ORR in the sunitinib arm failed to be significantly better than that in the placebo arm (32% *vs* 34%; P = 0.683). The study failed to demonstrate superiority for FOLFIRI plus suni-

tinib. Sunitinib plus FOLFIRI was associated with more grade ≥ 3 adverse events and laboratory abnormalities when compared to FOLFIRI plus placebo [neutropenia (68% *s* 30%), diarrhea (16% *vs* 8%), thrombocytopenia (11% *vs* 1%), anemia, stomatitis, fatigue, hand-foot syndrome and febrile neutropenia)]. In addition, more deaths as a result of toxicity (12 *vs* 4) and significantly more dose delays, dose reductions and treatment discontinuations occurred in the sunitinib arm[108].

A phase Ⅱ, open-label, single-arm study (NCT00668863) investigated oral sunitinib (37.5 mg/d 4 wk on, 2 wk off) combined with intravenous FOLFIRI (every 2 wk) for the first-line treatment of Japanese patients with unresectable or metastatic CRC. Median PFS was 6.7 mo by independent review and 7.2 mo by investigator assessment. ORR was 36.6% by independent review and 42.3% by investigator assessment. There was a high incidence of adverse events such as neutropenia (97.2%), leukopenia (97.2%); thrombocytopenia (84.5%), diarrhea (78.9%), nausea (78.9%), decreased appetite (74.6%) and fatigue (66.2%). Furthermore, almost 20% of patients discontinued study treatment permanently, due to adverse events and over 90% of required temporary interruptions of study treatment to perform treatment for related toxicities. The study was closed early when the concurrent phase Ⅲ study of first-line sunitinib plus FOLFIRI in non-Japanese patients with mCRC was stopped due to futility, as discussed previously.

Vatalanib

Vatalanib (PTK 787/ZK 222584; PTK/ZK) is a potent, orally active angiogenesis inhibitor that interferes with the kinase activity of all three VEGF receptors, acting as a competitive inhibitor at the ATP-binding site of the receptor kinase. This inhibition is reversible, highly selective for VEGFRs and translates to growth inhibition in a variety of different experimental tumor models. Although tumor regression did not occur, an attenuation of tumor growth was observed[109].

In the clinical phase Ⅲ trial CONFIRM1 (NCT00056459), 1168 patients with untreated mCRC were randomly assigned 1:1 to receive FOLFOX4 plus vatalanib or placebo. This study showed that the addition of vatalanib to FOLFOX4 did not improve PFS (7.7 mo in vatalanib arm and 7.6 mo in placebo arm: HR = 0.88, P = 0.118) or OS (21.4 mo in vatalanib arm and 20.5 mo in placebo arm: HR = 1.08, P = 0.260) and no statistically significant differences between the two treatment groups were observed in ORR (42% in vatalanib arm and 46% in placebo arm). Furthermore, vatalanib increased toxicity and more patients withdrew from treatment because of events other than disease progression in the vatalanib arm. Incidence of adverse event was 85.3% in vatalanib group and 77.5% in placebo group, particularly neutropenia, hypertension, and diarrhea. Concerning grade 3 or higher adverse events, the most notable differences were noted for hypertension (23.0% *vs* 6.8%, respectively), diarrhea (15.4% *vs* 11.1%, respectively), dizziness (7.4% *vs* 2.3%, respectively), and pulmonary embolism (5.7% *vs* 1.7%, respectively)[110].

The CONFIRM 2 (NCT00056446) was a phase Ⅲ trial aimed to compare treatment with vatalanib plus FOLFOX4 versus placebo plus FOLFOX4 in patients with previously treated mCRC, whose disease had recurred or progressed during or within 6 mo of treatment with irinotecan in combination with a fluoropyrimidine. The median OS was 13.1 and 11.9 mo (HR = 1.00, P = 0.957). Median PFS was longer with vatalanib than with placebo (5.6 and 4.2 mo, respectively; HR = 0.83, P = 0.013). Treatment-related adverse events occurred in 81.4% patients in vatalanib arm and in 71% of those in placebo arm. The most common grade 3 or higher side effects related to vatalanib were neutropenia, hypertension, diarrhea, fatigue and nausea[111].

CONCLUSION

Nowadays, mCRC treatment remains a challenge for oncologists worldwide. Over last three decades, mCRC treatment has came from fluropirimidine based chemotherapy to the addition of innovative chemotherapies regimen combination, such as FOLFOX, FOLFIRI, XELOX, XELIRI, 5-FU + LV, and innovative biologic therapies, such as bevacizumab, cetuximab and panitumumab. More recently, Aflibercept was approved for combination with standard chemotherapy in second line regimens for mCRC patients. Therefore, many options are now available with a powerful capacity to improve survival for metastatic patients. Thus, we should be aware for those previous mentioned innovative opportunities to fit them for each patient according to the adequate indication and tolerability. Also, pharmaco-economic studies are warranted to provide useful tools for public health entities, which might allow better clinical decisions, especially when willing those advances in research.

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REVIEW

Alcoholism and liver disease in Mexico: Genetic and environmental factors

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Abstract

Alcoholism and cirrhosis, which are two of the most serious health problems worldwide, have a broad spectrum of clinical outcomes. Both diseases are influenced by genetic susceptibility and cultural traits that differ globally but are specific for each population. In contrast to other regions around the world, Mexicans present the highest drinking score and a high mortality rate for alcoholic liver disease with an intermediate category level of per capita alcohol consumption. Mexico has a unique history of alcohol consumption that is linked to profound anthropological and social aspects. The Mexican population has an admixture genome inherited from different races, Caucasian, Amerindian and African, with a heterogeneous distribution within the country. Thus, genes related to alcohol addiction, such as dopamine receptor D2 in the brain, or liver alcohol-metabolizing enzymes, such as alcohol dehydrogenase class I polypeptide B, cytochrome P450 2E1 and aldehyde dehydrogenase class 2, may vary from one individual to another. Furthermore, they may be inherited as risk or non-risk haplogroups that confer susceptibility or resistance either to alcohol addiction or abusive alcohol consumption and possibly liver disease. Thus, in this era of genomics, personalized medicine will benefit patients if it is directed according to individual or population-based data. Additional association studies will be required to establish novel strategies for the prevention, care and treatment of liver disease in Mexico and worldwide.

Key words: Alcohol; Genes; Alcoholism; Alcohol dependence; Alcohol addiction; Alcohol abuse; Alcoholic liver cirrhosis; Anthropology

Core tip: Alcoholism and liver disease are leading global health problems. However, the severity and outcome of liver disease appear to vary between individuals and populations. In the present review, we analyze the general scope of alcohol consumption and its relationship with the pattern of drinking score in different countries. We focus on the development of alcoholism in Mexico, which has a strong historical background, and emphasize the need to understand the genetic and environmental factors affecting each population or geographical region of the world.

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INTRODUCTION

The human history of alcohol consumption has been documented for several thousand years[1]. Alcohol was undoubtedly the result of a fortuitous coincidence that occurred when fruits, grains and flower stalks were fermented for a long time. People may have begun to experience pleasure and happiness after tasting alcoholic beverages[1,2].

Alcoholic beverages are obtained from different sources, depending on the region of the world. Traditionally, must and wines are produced from grapes of the Middle East and Europe, whisky is made from various grains and sake is obtained from rice in Asia. In Mexico, "pulque" was introduced first, followed by tequila, which are made from the maguey and agave plants, respectively[3,4].

Historically, alcohol-based beverages have served as a source of needed nutrients and have been widely used for their medicinal, antiseptic and analgesic properties. However, during the last century, alcohol abuse has increased in several countries, thereby augmenting the rate of accidents and liver diseases. The range of liver diseases secondary to alcohol consumption is extensive, including acute alcoholic hepatitis, alcoholic liver disease (ALD), cirrhosis and hepatocellular carcinoma[5]. Different factors may affect the development of alcoholic liver damage, including the dose, duration and type of alcohol consumption, drinking patterns, gender and ethnicity[1,6-8]. Other associated risk factors include obesity, iron overload, concomitant viral hepatitis infection[1,7,9] and genetic factors[7,8]. Nonetheless, the degree of the association among alcohol consumption, morbidity and mortality due to ALD varies among individuals and populations worldwide. Alcohol consumption and ALD are linked to specific genetic and environmental factors that are prevalent in each population. However, which factors and how they are involved in both alcohol addiction and the adaptation of hepatic genes capable of metabolizing large amounts of ethanol without developing liver disease are challenging questions.

In this comprehensive review, we revisit the information on the worldwide consumption of alcohol and patterns of drinking associated with liver disease, emphasizing the history of alcoholism in Mexico and the differences in the genetic and environmental backgrounds with respect to alcoholism and liver disease among the different countries, with a focus on the genetic factors involved in alcohol dependence and alcohol abuse as well as liver-metabolizing enzymes.

WORLDWIDE ALCOHOL CONSUMPTION

The World Health Organization (WHO) published the total adult per capita alcohol consumption (liters of pure alcohol consumption/year) by distinct geographical regions of the world[10]. Three primary categories, high (10-12 L and > 12 L), intermediate (7.5-9.99 L and 5-7.49 L) and low (2.5-4.99 L and < 2.5 L), were created to compare alcohol consumption among different countries.

The countries with the highest alcohol consumption are located primarily in Europe (Czech Republic, United Kingdom, Ireland, Germany, France, Portugal and the Russian Federation) but also in other regions, such as South Korea, Australia, Nigeria, Uganda and Argentina. The intermediate category includes countries located in the Americas, such as the United States, Canada, Mexico, Chile, Brazil and Colombia, a few African countries, such as Cameroon, South Africa, Namibia and Botswana, and Norway in Europe. The low alcohol consumption category includes several countries within the Eastern Mediterranean region and Asia, generally representing those countries where religious beliefs prohibit alcohol consumption.

However, there have been different trends in the last 50 years regarding alcohol consumption in countries worldwide. Although several countries have increased alcohol consumption, others have decreased alcohol consumption (Figure 1). Furthermore, since 2008, the WHO has been in the process of drafting a global strategy to reduce the harmful use of alcohol[11]. These observations led us to analyze the effectiveness of these strategies to avoid or decrease alcohol consumption and to improve the understanding of the biological and social events involved in the drinking habits of alcohol in Mexico compared with other regions of the world.

MORTALITY DUE TO ALD

To address these concerns, we examined the mortality related to ALD within several countries. Interestingly, there is a discrepancy between mortality related to ALD and the per-capita alcohol consumption[12]; *e.g.*, Mexico is one of the countries with the highest mortality rate due to ALD but is not included among the countries with the highest alcohol consumption[12]. However, global comparisons among different populations are limited because not all countries report mortality related to ALD[12].

PATTERN OF DRINKING SCORE

The pattern of drinking score is a composite scale that ranges from 1 to 5 and focuses primarily on the degree of risk associated with how the alcohol is consumed rather than the amount of alcohol consumed. To build this scale, the following indicators are used: quantity of alcohol consumed by occasion, festive drinking, proportion of drinking events that involve becoming drunk, proportion of drinkers who drink daily, drinking with meals and drinking in public places[13].

Unlike alcohol consumption, which is measured by the amount of pure alcohol per capita/year, the pattern of drinking score is closely related to ALD. For example, the countries with the highest pattern of drinking score

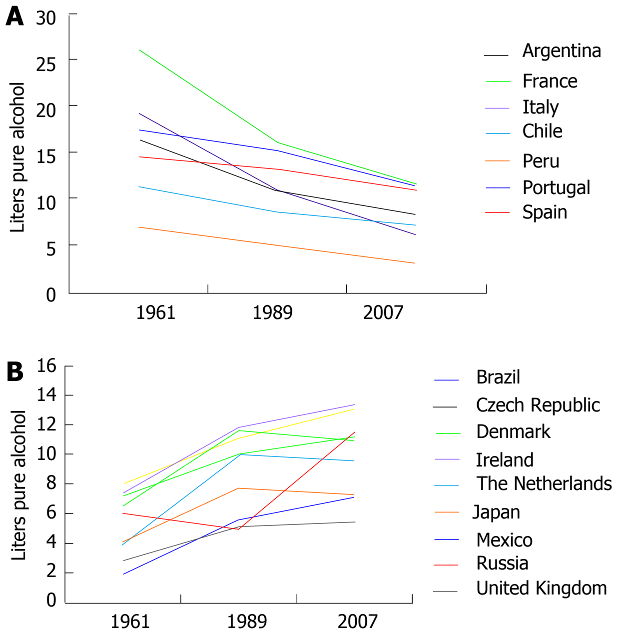

Figure 1 Trends of per-capita alcohol consumption in different countries. A: Countries that decreased consumption per capita since 1961 to 2007; B: Countries that increased consumption per capita since 1961 to 2007.

Figure 2 The tale of Mayahuel. The ancient gods gathered in the heavens understood that their people got bored eating only maize and chili; thus, they sent Quetzalcóatl, the god of the winds, to bring the young and beautiful goddess Mayahuel, granddaughter of a "tzintzimitl, a star who attempted to prevent the sun from rising". Quetzalcóatl and Mayahuel fell in love and together promised that they would give their people a magic plant to recover their happiness. Meanwhile, the evil grandmother noticed that Mayahuel had disappeared; thus, she and other "tzintzimitl" went down to the earth to find her. Mayahuel and Quetzalcóatl were hidden in the form of a tree with two arms (branches); one arm was Mayahuel, and the other was Quetzalcóatl. When the grandmother found them, she cut Mayahuel´s arm into many small pieces, but not Quetzalcóatl's, who was then transformed into a human again. Nothing could be done for Mayahuel, so Quetzalcóatl buried the leftover pieces in the ground and wept for his loss deeply. Finally, from these parts, the maguey was born. The mature maguey "cries" the honey-water, or "aguamiel", that emerges from the center of the plant, representing the tears of Quetzalcóatl. "Octli poliuhqui" is the fermented nectar of the maguey that brings happiness. Thus, Mayahuel in the Nahuatl language stands for all that surrounds the maguey[16,17]. Mayahuel may be considered a dual deity. On the one hand, she represented a woman with many breasts who nourished many children, the 400 rabbits ("Centzon Totochtin"); thus, she was associated with the earth and fertility. On the other hand, she was associated with drunkenness and adultery. This mythological symbol had such great influence that one day of the month was devoted to the rabbit, and those born on that specific date were destined to either be a drunk or commit adultery[17,18].

are Kazakhstan, Mexico, the Russian Federation, South Africa and Ukraine, and the countries with a lower-risk pattern of drinking are Portugal, Spain, France, Italy and Germany[13].

Taken together, alcohol consumption indicators, mortality rates and pattern of drinking scores, which all may contribute to ALD, are heterogeneous worldwide[12,13]. Thus, because ALD is a multifactorial problem, researchers should consider the anthropological and historical aspects prevalent among the different societies.

ALCOHOL CONSUMPTION IN MEXICO

Early history of alcohol consumption

To understand the interaction between the evolutionary and genetic changes associated with specific environments, it is necessary to know when and how these events occurred among the different populations. In the case of Mexico, the establishment of a sedentary lifestyle required approximately 5000 years[14,15]. During this period, the Mesoamericans began the domestication of the well-known staple foods of Mexico, such as maize (*Zea mays* L.), beans (*Phaseolus* spp), squash and pumpkin (*Curcubita* spp) and chili (*Capsicum* spp). This process was accompanied by the discovery and consumption of fermented alcoholic beverages made from a number of endemic agave plants (*Agave* spp). The origin of alcoholic beverages, as described by the Aztecs, was a mythical love story between two deities, "Mayahuel" and "Quetzalcóatl" (Figure 2)[16-18].

The core of the mature agave plant produces a honey water, or "aguamiel", rich in amino acids and proteins[19], which once fermented, produces the traditional alcoholic beverage. The Nahuas in their native language named the former "iztac octli" and the latter "octli poliuhqui"[20]. However, when the Spaniards arrived on the continent, "octli poliuhqui" was phonetically derived as the term "pulque"[20,21].

The "octli" but not "octli poliuhqui" served as nourishment for the elderly and sick and for women after childbirth[22]. The "octli" and perhaps the "octli poliuhqui" were given to all family members, including babies and children, in public ceremonies[17]. The "octli poliuhqui" was also used for medicinal purposes as an antidepressant or as an anesthetic before human sacrifice[16,18].

Additionally, the early Mexicans were familiar with the effects of the abuse of "octli poliuhqui"; thus, excessive drinking was strictly prohibited by law primarily during the religious holidays, and a death penalty was implemented[17]. The Aztec rulers often declared that the abuse of "octli poliuhqui" was the source and beginning of all evil and all ruin[17]. Unfortunately, these laws were not reinforced after the 15th century, granting a tolerance of the abusive consumption of alcoholic beverages during the colonial period[4,23].

The rich history of the consumption of "pulque" by the Mexicans over many centuries is an essential compo-

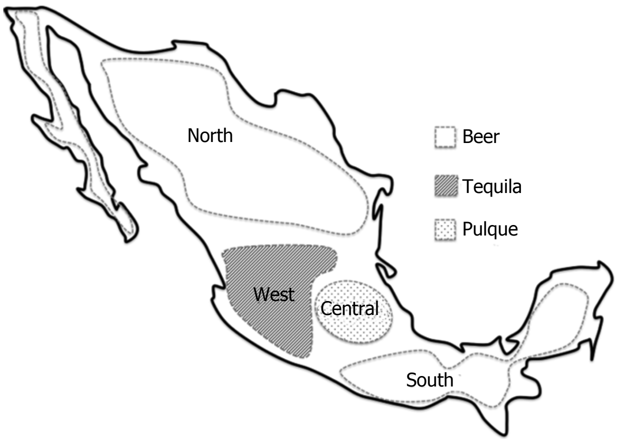

Figure 3 Main types of alcoholic beverages consumed by geographical region in Mexico.

nent of the framework that is required to understand the relationship among the Mexican genome, alcoholism and liver disease at present.

Alcohol consumption in Mexico at present

Mexico is one of the leading countries with a high mortality rate due to liver diseases in the world[10]. The National Health Secretariat reported an average of 25000 cases of cirrhosis per year from 2000 to 2010[24]. The primary etiologies of cirrhosis are alcohol, followed by hepatitis C infection and non-alcoholic steatohepatitis[25-27].

For Mexico, the WHO reported that the amount of alcohol consumed is 8.4 L of pure alcohol per capita among individuals older than 15 years of age, which corresponds to an intermediate category as previously described[10]. However, if this parameter is applied only to drinkers, alcohol consumption increases to 27.1 L, which is similar to what had been reported in countries with the highest levels of alcohol consumption per capita[10].

However, the pattern of drinking score shows a better scope of alcoholism among the Mexican population. By examining the amount of alcohol consumed by occasion, we observed that alcohol consumption occurs primarily during the weekends[28], unlike in Europe where they drink wine almost daily, at lunch or dinner.

Hepatologists may advise their patients not to drink any alcoholic beverage to maintain a healthy liver. However, a large proportion of adults around the world drink alcoholic beverages[1,5,6]. Thus, the recommendation to avoid liver damage is that the amount of alcohol consumed should be equal or less than 2 drinks per occasion (20-40 g ethanol), not more than 4 drinks per day and not more than 10 to 12 drinks per week, allowing the liver to rest at least 1 or 2 d[1,5,6]. Furthermore, it has been suggested that the number of alcoholic drinks should be less in women than in men because women have a higher risk for developing ALD[1,5,6].

However, each weekend, approximately 30 million Mexicans have been estimated to consume more than five drinks per occasion (more than 80 g of ethanol), with another 10 million consuming at least one alcoholic drink daily. However, alcohol abuse has been detected in 5 million people with a strong dependence on alcohol[28].

The average Mexican begins consuming alcohol before the age of 18 years perhaps because of a strong cultural influence. Studies conducted in the western region of Mexico have shown that 61.4% of the 12- to 17-year-old have already begun to drink alcohol[27]. The primary types of alcoholic beverages consumed in Mexico are beer, tequila and "pulque", and other distilled beverages are consumed in a lower proportion[27-30]. However, the distribution of alcoholic beverage preferences is heterogeneous. Thus, in central Mexico, "pulque" is preferred, in contrast to tequila in the west or beer in the northern and southern parts of the country (Figure 3). These preferences are associated with the historical cultural background of each region and may be related to the mortality caused by cirrhosis. The mortality rate in central Mexico is greater than 30/100000, followed by the north at less than 10/100000 and the west at less than 5/100000[30,31].

In western Mexico, young people begin to drink beer either during the weekend or at any social or religious event, such as weddings, coming-of-age parties and christenings. After the initiation of alcohol use, the number of beers consumed per occasion over the weekend ranges from 4 to 6 (80-100 g); this number gradually increases to 20-24 beers/355 mL each (300-360 g of alcohol)/occasion per person over a period of approximately 10 years. The second stage involves the combination of beer with tequila or any other distilled beverage during a period of 8 to 10 years. During this stage, the amount of alcohol consumed ranges from 380 to 640 g daily. In the third stage, alcohol dependence is severe, and patients may or may not present with cirrhosis. By this time, they drink an average of 510 g of alcohol per day (450-720 g)[26,32-34].

The time between the initiation of alcohol use and the diagnosis of cirrhosis is 23 to 30 years[26,35]. However, we have identified two distinct age peaks of clinical cirrhosis. In the first group, patients are young, approximately 30 years old, and a plausible genetic predisposition to liver cirrhosis has been proposed to be involved. In the second group, the average age is approximately 45 years[33]. Compared to other countries, Mexico, according to our findings, may have the youngest people with alcoholic cirrhosis in the world. Apparently, the Apo E2[33] and FABP2[36] gene polymorphisms may be involved in the early onset of ALD among the Mexican population.

Clinical profile of Mexican patients with ALD

The majority of patients with ALD seek medical attention in the advanced stages of the disease with a Child-Pugh score of C and multiple complications, such as encephalopathy, variceal bleeding, infections and ascites[25,27,35]. These clinical characteristics are present in the two primary age groups of patients with alcoholic cirrhosis[33]. Furthermore, the patients with alcoholic cirrhosis continue to drink high amounts of alcohol after diagnosis

and may die earlier in life due to clinical complications[25]. This observation may be one of the foremost reasons why hepatocellular carcinoma is rare in Mexico compared with other regions of the world[37,38], in conjunction with other environmental factors[39].

ALD has been associated with nutritional deficiencies and malnutrition worldwide[40]. However, preliminary data from a reference center in western Mexico have shown that obesity is also present. Among 90 patients, 17% of the alcoholic cirrhotic patients were malnourished, whereas overweight and obesity were detected in 33% of the patients, with another 50% of normal weight[35]. These data are consistent with the fact that Mexico has the highest prevalence of obesity[41], thus adding a new risk factor for liver disease. Furthermore, in this group of patients, 34% of the patients had drug addictions, which is an increasing social and health problem[35].

Thus, the combination of alcoholism, obesity, drugs and, in several cases, viral hepatitis B or C, leads us to explore specific strategies for treatments and prevention programs to detect cirrhosis at early stages of the disease.

GENETICS OF ALCOHOL DEPENDENCE OR ALCOHOL ABUSE

In recent decades, researchers have been using various strategies to identify genes that may be associated with alcohol dependence or alcohol abuse. Studies based on candidate genes[42-45] or linkage disequilibrium were followed by the advances in genotyping that have resulted in the widespread use of genome-wide association studies[46,47]. Previous studies in families, twins and adoption studies have shown that approximately 40%-60% of the variance in the risk for developing alcoholism can be explained by genetic factors[43-47]. However, the interactions between genes and several environmental factors have led experts in the field to identify at least two types of alcoholism: (1) a more severe, more genetic and early-onset type of alcoholism; and (2) a less severe, more environmental and late-onset type of alcoholism[48-52].

Regarding the role of genetic factors in the susceptibility to alcohol dependence and alcohol abuse, research has primarily aimed to study the expression of brain and liver genes. For example, the major brain genes that modulate the neuroadaptative mechanism that translates alcohol stimuli into pleasure, anxiety or cravings are opioid receptor mu 1[53-55], catechol-*O*-methyltransferase[56], γ-aminobutyric acid receptor A[57,58], 5-hydroxytryptamine (serotonin) receptor adenylate cyclase-coupled[59,60], cholinergic receptor muscarinic 2[61,62], vesicular monoamine transporter 2[63-65] and dopamine receptor D2[48,66-68].

In the liver, several alcohol dehydrogenase (ADH) enzymes, primarily alcohol dehydrogenase class I polypeptide B (ADH1B)[52], cytochrome P450 2E1 (CYP2E1)[69] and aldehyde dehydrogenase class 2 (ALDH2)[52,70], and other minor ADHs, such as ADH1C[71] and ADH4[72], have been related to alcohol metabolism and alcoholism. The three major enzyme genes express variants with different catalytic activities (V_{max}) and Michaelis constants (K_m); thus, their ability to metabolize substrates is variable.

The combination of the allelic profile of these brain and liver genes may affect the risk of or protection against alcohol dependence or alcohol abuse as well as the amount of alcohol metabolized in the liver and the susceptibility to liver damage. Variances in the distribution of these gene polymorphisms may mark phenotypic differences among populations for the aforementioned features. Hence, for this review, the biological functions of dopamine receptor D2 (DRD2), ADH1B, CYP2E1 and ALDH2 are briefly described, and their global allelic frequencies are compared, including those reported for the Mexican population.

DRD2

Alcohol has a stimulatory effect on the dopaminergic neurons of the ventral tegmental area. Dopamine is captured by DRD2 in these neurons in the nucleus accumbens, causing a pleasant effect that is integrated into the mesolimbic system[48,67,68].

The DRD2 *Taq I A1* polymorphism consists of a T/C nucleotide substitution (rs1800497) that alters the Taq I restriction site located 10541 bp downstream of the termination codon. Several studies have investigated the association of this gene polymorphism with alcohol dependence. *Taq I A1* allele carriers reportedly have lower amounts of DRD2 receptors than the Taq I A2 carriers[73]. Thus, *A1* allele patients require higher amounts of alcohol to achieve the desired effect[66-68,74-76]. In additional studies, the association between the *A1* allele Taq I and alcohol use disorders has been corroborated in some but not in others. However, in several meta-analyses, a significant association between Caucasian *A1* allele carriers and alcohol addiction has been found[48,77].

The allelic distribution of DRD2 displays a wide range of frequencies worldwide[74-79], but the highest prevalence of the *A1* allele is found among the Amerindian Pima (83%) and Mayas (71%) from Mexico[80,81] (Figure 4).

ADH1B

The *ADH1B* gene has a polymorphic site, resulting in the Arg47His substitution (rs1229984). The *A2* allele (ADH1B His47) confers a 100-fold higher catalytic activity to the ADH1B enzyme than the *A1* allele (ADH1B Arg47). The *A2* allele carriers have a higher ethanol oxidation capacity than the A1 carriers. However, the A2 carriers have a higher acetaldehyde production that leads to an alcohol-flushing response that has been considered to be protective.

The protective effect of the *A2* allele against alcohol dependence is well known in the East Asian population[82]. A study conducted in a cohort of pregnant women from England demonstrated that the A2 carriers consumed less alcohol before pregnancy, had less incidents of binge drinking during pregnancy and were abstainers during the first trimester of gestation[83]. However, al-

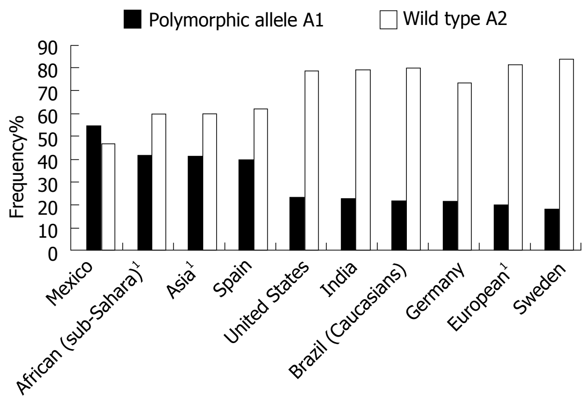

Figure 4 Frequency of Taq I A dopamine receptor D2 polymorphisms in different countries. [1]HapMap.

though this allele apparently reduces the risk of alcohol dependence, it may confer a substantial risk of chronic liver disease, especially among heavy drinkers[84].

In contrast, *A1* allele carriers do not present with the rapid production of acetaldehyde, which eliminates the alcohol-flushing response. A study conducted in a large cohort of individuals from Copenhagen revealed that *A1* allele homozygotes drank more alcohol and had a higher risk for developing alcohol dependence[85]. The *A1* allele has also been associated with alcohol dependence in several other studies[86-88].

CYP2E1

The *CYP2E1* gene, which encodes the enzyme that oxidizes ethanol in the microsomal oxidation system, is essential in the pathophysiology of ALD. Alcohol consumption induces the expression of CYP2E1, which is directly involved in the primary oxidation pathway of this substrate, displacing the enzymatic activity of ADH1B (K_m: 8-10 mmol/L for CYP2E1 *vs* 0.2-2.0 mmol/L for ADH1B)[69,89].

The *C2* allele, which is less common, has a C/T transition at nucleotide position -1019 within the 5' terminal regulatory region. The *C2* allele is associated with a 100-fold higher transcription activity, higher protein concentration and increased enzyme activity, which lead to a faster rate of alcohol oxidation[90,91].

The *C2* allele carriers have been demonstrated to consume excessive amounts of alcohol, which may be caused by the high transcriptional activity of CYP2E1. *C2* allele carriers metabolize ethanol (alcohol) to acetaldehyde at a higher rate. Acetaldehyde is a highly toxic and mutagenic metabolite that increases oxidative stress by producing reactive oxygen species and lipid peroxides, such as 4-hydroxy-2,3-nonenal, 4-hydroxy-2,3-alkenals and malondialdehyde[92]. An association between an increased risk for ALD and alcoholic cirrhosis has been reported among the carriers of the *C2* allele[93-95], several of whom belong to the mestizo population of western Mexico[96].

ALDH2

The *ALDH2* gene encodes the primary mitochondrial isoform enzyme that oxidizes acetaldehyde to acetate in the liver[70,97]. The C/G transition in exon 12 of ALDH2 causes an amino acid substitution of glutamic acid for lysine at position 487 (ALDH2 Glu487Lys, rs671). The *A2* allele (ALDH2 Lys487) has little or null enzymatic activity. This deficiency leads to the accumulation of acetaldehyde and consequently provokes a flushing response, which discourages alcohol drinking[97]. Because flushing is an undesirable symptom, it confers relative protection against abusive alcohol consumption. An association between *A2* allele carriers and a lower risk for alcohol dependence and reduced alcohol use has been reported[98].

With regard to the distribution of the polymorphisms of the liver alcohol-metabolizing genes, all present contrasting frequencies among different population groups (Figure 5A-C). Among Asians, the *ADH1B* gene displays the highest frequency of the *A2* protective allele, with 78% in Japan and 69% in China. In contrast, the lowest frequency for the *A2* allele was detected in Germany and Mexico, at 4% and 3%, respectively (Figure 5A)[91,99-105], wherein the frequency of the *A1* allele associated with alcohol dependence was much higher.

The *C2* allele for the *CYP2E1* gene has a frequency of approximately 30% in Japan and China and 2% in the United States. In Chile and Mexico, the frequency is 16% among the mestizo population (Figure 5B)[91,93,94,105-108]. Interestingly, among the Amerindians of western Mexico, such as the "Huichol" people, this gene polymorphism shows a prevalence of 50%, which is the highest rate reported to date[91].

The highest frequency of the *A2* allele for the *ALDH* gene has been reported in China (29%) and Japan (26%). In Germany, Sweden and Mexico, its frequency is extremely low or absent[91,99-105], which could explain, to some extent, the high amount of alcohol consumption that has been reported in those countries.

We could speculate that the selective evolution of both the brain and liver genes was not necessarily directed only by the exposure to alcohol. For example, liver cytochrome genes metabolize a large variety of xenobiotics, whereas those expressed in the brain fulfill the addiction criteria. However, an alternative point of view is to consider these genes as part of a general survival mechanism. Thus, the basic biological necessities of life, such as food (sugars, *e.g.*, glucose) or sexual reproduction, are ensured and rewarded by pleasure; however, these necessities may not be driven by pleasure exclusively.

CONCLUSION

At the end of the last century, we began to understand how liver genes are involved in the metabolism of ethanol and how cerebral genes are related to addictions. Additional genetic studies, including genome-wide association studies, will corroborate the association of specific alleles with alcoholism and ALD. The next step may be a personalized medicine strategy for the prevention, diagnosis and treatment of liver diseases. However, as aforementioned in this review, genes and environmental

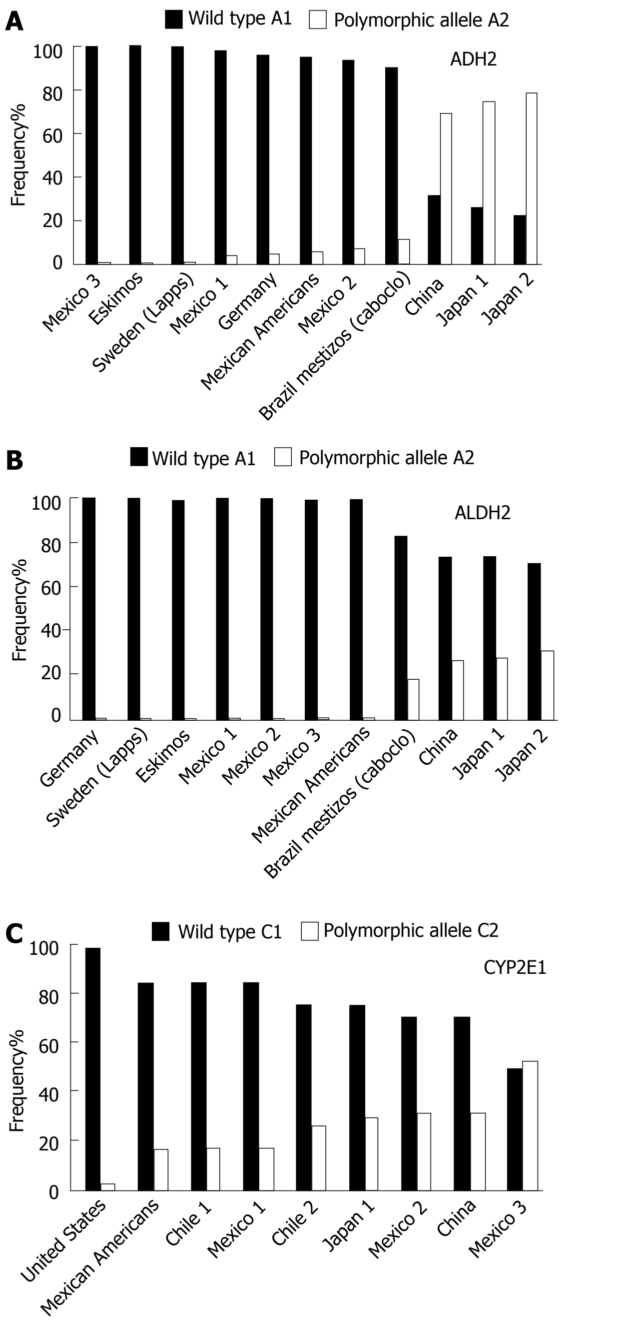

Figure 5 Frequency of polymorphisms of enzymes that metabolize alcohol. A: Alcohol dehydrogenase 2 (ADH2); B: Aldehyde dehydrogenase class 2 (ALDH2); C: Cytochrome P450 2E1 (CYP2E1) in different countries. A-B: Mexico 1: Western Mexico; Mexico 2: Native from Mexico "Otomi"; Mexico 3: Native from Mexico "Huicholes"; Japan 1: Rural country; Japan 2: Mestizos from Japan; C: Mexico 1: Western Mexico; Mexico 2: North an central Mexico; Mexico 3: Native from Mexico "Huicholes"; Chile 1: Mestizos; Chile 2: Native from Chile "Mapuches".

factors are involved in the development of ALD, which requires an in-depth analysis of the different populations. Therefore, the data found in several regions of the world may not correlate to populations from different geographic areas.

Mexicans are an admixture population that has inherited specific alleles from different races, predominantly Caucasian, Amerindian and African[109,110]. Based on the current data on allelic frequencies in different countries, the Mexican population has a particular genetic profile that may explain the epidemiological and clinical manifestations of alcohol-related liver diseases. Thus, the expectation that the different allelic variants of the aforementioned genes (*DRD2*, *ADH2*, *CYP2E1* and *ALDH2*) will express themselves individually is plausible. However, considering these alleles as a haplogroup may generate risk or non-risk phenotypes related to liver disease, as well as to proneness towards or resistance against the high intake of alcohol. Haplogroups that could confer a non-risk phenotype for alcoholism and liver damage could be *DRD2*A2*, *ADH2*A2*, *CYP2E1*C1*, *ALDH2*A2* and *DRD2*A2*, *ADH2*A1*, *CYP2E1*C1*, *ALDH2*A2* because they are related to non-addiction plus flushing by the accumulation of acetaldehyde, exhibiting a protective effect. The haplogroups that could confer risk phenotypes for alcoholism and liver damage could be *DRD2*A1*, *ADH2*A1*, *CYP2E1*C1*, *ALDH2*A1* and *DRD2*A1*, *ADH2*A1*, *CYP2E1*C2*, *ALDH2*A1* because these are related to addiction plus the efficient metabolism of alcohol but exposure to acetaldehyde. This observation may explain why some patients who consume heavy amounts of alcohol per day (> 80 g/d) for more than 20 years do not have liver damage, whereas others with a less than or equal to consumption level and less exposure suffer liver damage and even die from cirrhosis or its complications[1,6,7]. However, additional studies are required to demonstrate the association between these hypothetical allelic profiles and the clinical outcomes of alcohol-dependent patients in Mexico and worldwide.

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REVIEW

Management of post-hepatectomy complications

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Abstract

Hepatic resection had an impressive growth over time. It has been widely performed for the treatment of various liver diseases, such as malignant tumors, benign tumors, calculi in the intrahepatic ducts, hydatid disease, and abscesses. Management of hepatic resection is challenging. Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity and mortality. Especially, complex resections are being increasingly performed in high risk and older patient population. Operation on the liver is especially challenging because of its unique anatomic architecture and because of its vital functions. Common post-hepatectomy complications include venous catheter-related infection, pleural effusion, incisional infection, pulmonary atelectasis or infection, ascites, subphrenic infection, urinary tract infection, intraperitoneal hemorrhage, gastrointestinal tract bleeding, biliary tract hemorrhage, coagulation disorders, bile leakage, and liver failure. These problems are closely related to surgical manipulations, anesthesia, preoperative evaluation and preparation, and postoperative observation and management. The safety profile of hepatectomy probably can be improved if the surgeons and medical staff involved have comprehensive knowledge of the expected complications and expertise in their management. This review article focuses on the major postoperative issues after hepatic resection and presents the current management.

Key words: Hepatectomy; Postoperative complication; Management

Core tip: Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity and mortality. Common post-hepatectomy complications include fever, hemorrhage, bile leakage, liver failure, pleural effusion, and subphrenic infection. The aim of this study was to summary the causes for post-hepatectomy complications and to discuss the prevention and treatment trick for postoperative complications.

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INTRODUCTION

The era of hepatic surgery began with a left lateral hepatic lobectomy performed successfully by Langenbuch in Germany in 1887. Since then, hepatectomy has been widely performed for the treatment of various liver diseases, such as malignant tumors, benign tumors, calculi in the intrahepatic ducts, hydatid disease, and abscesses.

Operation on the liver is especially challenging because of its unique anatomic architecture and because of its vital functions. Despite technical advances and high experience of liver resection of specialized centers, it is still burdened by relatively high rates of postoperative morbidity (4.09%-47.7%) and mortality (0.24%-9.7%)[1-16] (Table 1). Common post-hepatectomy complications include fever, hemorrhage, bile leakage, liver failure, pleural effusion, and subphrenic infection, which we will discuss.

POSTOPERATIVE FEVER AND INFECTIONS

Venous catheter-related infection

Deep-vein catheterization is routinely performed for hepatic surgery, and venous catheter-related infection is the most common cause of fever after hepatectomy. This source of fever should be considered if the fever cannot be attributed to some other cause. If it cannot, the catheter should be immediately removed and its tip cultured, so that appropriate antibacterial therapy can be instituted promptly[17-21].

Pleural effusion

Reactive pleural effusion may occur after hepatectomy and usually is the result of diaphragmatic injury, obstruction of thoracic venous or lymphatic systems, or surgical manipulation on the hepatic coronary ligament (usually causing a subphrenic fluid collection). The pleural effusion, which most often occurs in the right chest, can cause fever even though it is aseptic. X-ray and ultrasound examinations should be performed promptly in febrile patients in order to determine whether a pleural effusion has developed. If only a small effusion is present it may spontaneously resolve, and if the patient has no significant symptoms or signs, no special treatment will be needed; otherwise, thoracic puncture and drainage of the effusion should be carried out[22-24].

Incisional infection

Incisional infection usually occurs within 1 wk after operation. Swelling and exudation at the incision site, or in the case of severe infection, dehiscence of the wound may be seen. If infection is found, the sutures and necrotic tissue should be removed and adequate drainage established. Antibiotics may be prescribed to help control the infection. If wound dehiscence is present, tension sutures may be placed[25-27]. Albumin may be administered intravenously in order to help relieve intra-abdominal pressure if present[28].

Pulmonary atelectasis or infection

Postoperative atelectasis or pulmonary infection most commonly presents 3-5 d after the operation. Symptoms and signs may include chest tightness, shortness of breath, and cyanosis. Surgical trauma, prolonged bed rest, and limited coughing because of incisional pain are the major factors predisposing to pulmonary atelectasis or infection. The findings of hypoxemia, determined by blood-gas analysis, and abnormalities seen on chest X-ray films, will assist in making the diagnosis. If the pulmonary infection progresses to pneumonia, the patient may have fever, cough, and pulmonary rales; increased bronchovascular shadows and pulmonary consolidation may be seen on chest X-ray films. Analgesic drugs may be given to relieve patient's pain and to facilitate deep breathing; bronchial lavage may be performed for relief of airway obstruction; and antibiotics may be prescribed after sputum culture and testing bacteria for drug sensitivity[28-30].

Ascites

Ascites is common in hepatectomy patients who have associated liver malfunction or cirrhosis. Ascitic fluid may drain from the incision site or the drainage tube[31]. Accumulation of much ascites may result in imbalance of water and electrolytes. Paracentesis for treatment of the ascites usually is not recommended; administration of diuretics and albumin is preferred. However, if the ascites is suspected of being infected and the source of fever, diagnostic paracentesis under ultrasonic guidance should be performed[32-35].

Subphrenic infection

Subphrenic infection is a severe complication of hepatectomy, usually resulting from incomplete or premature removal of a subphrenic fluid collection or a bile leak. Fever, tenderness in the upper abdomen, and abdominal muscle tension are the major manifestations of subphrenic infection. Septicopyemia or septicemia may develop if infection is severe, which may occur with pleural effusion or pulmonary atelectasis[36]. Thorough drainage of the fluid, in addition to anti-inflammatory therapy, is critical in the treatment. Ultrasonic guidance may be useful in the aspiration of subphrenic fluid collections or in the evacuation of abscesses. Open operation may be needed if the infection is severe[37].

Urinary tract infection

Fever, back pain and bladder irritation are the common symptoms of upper urinary tract infection. In contrast, fever is not common in lower urinary tract infection, which is usually manifested by dysuria and urinary frequency and urgency. Treatment of the urinary infection includes anti-inflammatory medications, oral hydration, and medications to relieve cystospasm and symptoms of bladder irritation.

POSTOPERATIVE HEMORRHAGE

Intraperitoneal hemorrhage

The incidence of intraperitoneal hemorrhage ranges from 4.2% to 10%[6,38-41]. Three common reasons for intraperitoneal hemorrhage are: (1) bleeding from the surfaces of the residual liver, which may be a consequence of arterial branch truncation or congestion of the hepatic vein due to stenosis or ligation; (2) incomplete intraoperative hemostasis, which sometimes is due to inappropriate

Table 1 Summary of studies investigating the post-hepatectomy mortality and morbidity

Ref.	Journal	Date of publication	Country of study	NO. of Patients studied	Disease's diagnosis	Mortality and morbidity of hepatectomy
Savage *et al*[1]	*Ann Surg*	December 1991	United States	300	Liver trauma or liver tumors	The operative mortality was 19% (1962-1979) or 9.7% (1980-1988), and the overall complication rate was 12.3%
Wu *et al*[2]	*Zhonghua Waike Zazhi*	May 2002	China	1762	Liver cancer	The total mortality was 0.40%, and the total complication rate was 4.09%
Ishikawa *et al*[3]	*Hepatogastroenterol*	November-December 2002	Japan	139	HCC	The mortality within 30 postoperative days was 2.2%, and complication morbidity was 40.2%
Descottes *et al*[4]	*Surg Endosc*	January 2003	France	87	Benign liver tumors	There was no postoperative mortality, and the postoperative complication rate was 5% (laparoscopic liver resection)
Dimick *et al*[5]	*Arch Surg*	January 2003	United States	569	Malignant or benign liver disease	The overall in-hospital mortality rate was 4.8%
Benzoni *et al*[6]	*Hepatobiliary Pancreat Dis Int*	November 2006	Italy	287	HCC or liver metastasis	In-hospital mortality rate was 4.5%, and the morbidity rate was 47.7%
Benzoni *et al*[7]	*Hepatogastroenterol*	January-February 2007	Italy	134	HCC	In-hospital mortality rate was 7.4%, and the morbidity rate was 47.7%
Mullen *et al*[8]	*J Am Coll Surg*	May 2007	United States	1059	Noncirrhotic patients	The complication rate was 43%, and the 90-d all-cause mortality rate was 4.7% (1.9% patients died of causes unrelated to the liver)
McKay *et al*[9]	*Ann Surg Oncol*	May 2008	Canada	1107	Liver tumor	In-hospital mortality rate was 6.0%, and an overall complication rate was 46%
Feng *et al*[10]	*World J Gastroenterol*	December 2008	China	827	Benign hepatic lesion	In-hospital mortality rate was 0.24%, and the postoperative complication rate was 13.54%
Tomuş *et al*[11]	*Chirurgia (Bucur)*	May-June 2009	Romania	50	Benign hepatic lesion	There was no mortality, and the morbidity rate was 18%
Cescon *et al*[12]	*Ann Surg*	June 2009	Italy	1500	Malignant or benign disease	Overall mortality was 3%, and the morbidity was 22.5%
Huang *et al*[13]	*Chin Med J (Engl)*	October 2009	China	2008	Malignant or benign liver disease	The overall hospital mortality was 0.55%, and the overall postoperative complication rate was 14.44%
Mathur *et al*[14]	*J Gastrointest Surg*	August 2010	United States	3960	Liver tumor	The overall mortality rate was 2.5%, and the overall complication rate was 23.3%
Sato *et al*[15]	*J Gastroenterol*	October 2012	Japan	5270	HCC	In-hospital mortality was 2.6%, and the postoperative complication rate was 14.5%
Dan *et al*[16]	*Chirurgia (Bucur)*	November-December 2012	Romania	133	Benign or malignant tumors	The overall mortality rate was 2.25%

HCC: Hepatocellular carcinoma.

manipulation of the hepatic vein root or trauma to the diaphragm, and increased intrathoracic pressure and vena cava pressure which may lead to bleeding; and (3) vascular sutures loosened or fallen off, an event which usually is ascribed to elevated pressure in the vena cava from patients' body movement, such as turning over or coughing severely. Detachment of the ligature on the short hepatic veins may cause a gap in the vena cava wall. Postoperative intraperitoneal hemorrhage usually occurs within 48 h, and from the residual liver's surface or the diaphragm. Thorough intraoperative hemostasis is critical and must be ascertained before the operation is concluded. When the root of the hepatic vein is manipulated during the operation, hemorrhage from the vein or anterior to the inferior vena cava should be carefully sought by increasing the intrathoracic pressure artificially. Mattress sutures with hepatic needles should be used for the hemostasis, and the traumatized surface can be covered with hemostasis film, gelatin sponge, biological glue, or omentum as means of achieving additional hemostasis[42]. The presence of persistent bloody drainage might indicate that intraperitoneal clots have formed, which may occlude abdominal drains, leading to abdominal distention. Close monitoring of vital signs and transfusion of whole blood, platelets, and plasma are usually recommended as long as the patient's blood pressure and pulse remain stable. Otherwise, secondary open surgery should be considered[43]. We recommend that open surgery to attain hemostasis be performed if blood loss exceeds 1000 mL/h for more than eight hours. In summary, correct timing for operations on infected liver sections, careful manipulation during operation, and thorough hemostasis and drainage are critical for success in attaining hemostasis.

Coagulation disorders

Five common causes of coagulation disorders associated with hepatectomy are: (1) functional failure of the residual liver due to prolonged ischemia, especially in the presence of cirrhosis[44]; (2) massive intraoperative bleeding, or blood transfusion of more than 4000 mL;

(3) consumption of coagulation factors and platelets due to severe infection; (4) overdose of heparin after hepatic artery or portal vein catheterization; and (5) cardiopulmonary bypass or extracorporeal circulation[45,46]. Coagulation time, prothrombin time, platelet count, and fibrinogen level should be tested to aid in making the diagnosis of a postoperative coagulation disorder, and the 3P test may be performed if necessary[47]. Expansion of the circulating blood volume and transfusion of fresh blood should be carried routinely once a coagulation disorder is confirmed, and prompt administration of fibrinogen, prothrombin complex, fresh platelets, and plasma cold precipitates also is important[48-50]. Protamine can be administered to neutralize the heparin if it has been overdosed.

Gastrointestinal tract bleeding

The common causes of gastrointestinal tract bleeding after hepatectomy are: (1) stress ulcer, the most common; (2) portal hypertension due to liver cirrhosis; and (3) congestion of gastrointestinal organs because of secondary portal hypertension due to the limited volume of the residual liver. Gastrointestinal tract bleeding usually occurs within two weeks after operation. It may be manifested by the passage of brown or bloody drainage, hematemesis, melena, deterioration of vital signs, and abdominal pain. If the bleeding is mild, nasogastric suctioning and administration of proton pump inhibitors and hemostatic drugs may be adequate treatment. Somatostatin and ulinastatin may be given if the bleeding is massive. Operation should be considered if the blood pressure and pulse are unstable, or if hemorrhage persists after 48 h of aggressive treatment[51-54].

Biliary tract hemorrhage

Iatrogenic bile duct injury is the most common cause of biliary tract hemorrhage after hepatectomy. Surgical maneuvers, including operating in the hepatic portal region, bile duct exploratory surgery, and placement of T-tubes in the biliary ducts could result in biliary tract hemorrhage. Other common causes are mucosal erosion ulcer and coagulation disorders due to biliary tract infection and inflammation. The major manifestations of biliary tract hemorrhage are right upper quadrant gripping pain, upper gastrointestinal bleeding, and obstructive jaundice. Usually, bleeding of this kind can be treated effectively with appropriate hemostasis, antibiotics, and supportive measures. For patients who have massive biliary tract bleeding or whose bleeding site is unclear (after hepatic artery angiography), explorative operation should be carried out[55-59].

BILE LEAKAGE

The incidence of bile leakage ranges from 4.0% to 17%[60-63]. Common causes of postoperative bile leakage are: (1) truncation of the distal bile duct in the residual liver, the most common cause; (2) leakage at the bile duct-intestinal anastomosis, or incomplete suture around the T-tube; and (3) injury of the bile duct from inappropriate surgical technique. A retrospective analysis by Yoshioka *et al*[64] of 505 hepatectomy cases found that the incidence of bile leakage was 6.7%, with three independent risk factors: (1) multiple hepatectomy (P = 0.002, OR = 3.439; 95%CI: 1.552-7.618); (2) traumatized liver surface ⩾ 57.5 cm^2 (P = 0.004, OR = 5.296; 95%CI: 1.721-16.302); and (3) intraoperative bleeding ⩾ 775 mL (P = 0.01, OR = 2.808; 95%CI: 1.280-6.160)[64]. Another analysis by Sadamori *et al*[65] of 359 hepatectomy cases found that operative time ⩾ 300 min was an independent risk factor for bile leakage after hepatectomy. To help predict if postoperative bile leakage will occur, the residual liver can be covered with wet gauze, which may show the presence of minimal bile seepage. To help avoid postoperative bile leakage, biological glue can be applied to the surface of the residual liver, and a C tube can be placed in the cystic duct for decompression[66,67]. Intraoperatively, bile leakage might be revealed with the use of indocyanine green fluorescein[68-70]. Close postoperative monitoring is mandatory and should include observing for abdominal pain, rebound tenderness, muscle tension, and bile leakage from the drainage tube. Bile leakage also may be evident by the presence of bile in the peritoneal drainage (the concentration of bilirubin in the bile will be higher than in serum). In addition, computed tomography (CT) visualization can be used to determine if the bile duct is occluded and, if so, where the occlusion is located. A drainage tube can remain in the bile duct if there is no sign of peritonitis; the bile leakage may resolve spontaneously within two months. However, if peritonitis develops, open surgery should be performed as soon as possible for thorough cleaning of the abdominal cavity and repair of the damaged common bile duct. Antibiotics may be administered for control of infection, and supportive treatment should be given as usual after a major operation[71,72]. It has been reported that bile leakage occurred in 14 of 96 patients who underwent hepatectomy; nine were treated successfully without operation, but five required a second operation. In general, non-operative treatment was sufficient if the results of ERCP and CT were negative for bile leakage, but operative intervention was needed if conservative therapy failed[73].

LIVER FAILURE

Liver failure is a severe postoperative complication of hepatectomy. It is closely associated with active hepatitis, cirrhosis, limited residual liver tissue, massive intraoperative hemorrhage, the mode and duration of hepatic portal vein occlusion, the kind of anesthesia used, and perioperative medication used. An incidence of liver failure after hepatectomy of about 0.70%-33.83% has been reported[74-77], and the failure was related to inadequate residual liver tissue and functional capacity[78,79]. Comprehensive therapy for liver failure includes postoperative supplementation with albumin, fibrinogen or prothrombin complex; intravenous nutrition; and transfusion of

fresh blood. Prognosis is poor if coagulation disorders develop. Presently, the most effective therapy for liver failure is liver transplantation, but it is associated with a high mortality rate in patients with liver cirrhosis and, therefore, it remains a controversial treatment choice in this circumstance[80-86], Generally, prevention of liver failure is felt to be more important than treatment of it. Some common preventive measures are: careful preoperative assessment of the liver's functional reserve and institution of measures to improve the liver function. Prevention of intraoperative bleeding and the need for blood transfusion also are important in preventing liver failure. In one report, the incidence of postoperative complications increased significantly when the intraoperative blood loss exceeded 1200 mL[11]. Several methods can be used to reduce the chance of intraoperative bleeding: CUSA[87-89], heat solidification technology[90-93], reduction of central venous pressure[94-96], and blocking of hepatic portal blood inflow (with or without control of hepatic blood outflow)[97-101]. For patients with liver cirrhosis, the volume of residual liver and the time of portal occlusion must be strictly assessed. Also, the method used for occluding blood flow to the liver must be appropriately selected. It has been recommended that half hepatic blood flow occlusion should be used for patients with cirrhosis, and hepatic blood inflow occlusion without hemihepatic artery control (hemi-hepatic artery-preserved portal occlusion) used if half occlusion is difficult or inadequate. Hepatic blood inflow occlusion without hemihepatic artery control is simple for operation, with less damage to the liver function; more importantly, the effect of the blood flow blocking is equivalent to the half-hepatic blood flow blocking[102,103]. The procedure for inflow occlusion is the following: the hepatic artery is exposed first, to separate the right and left hepatic arteries from the root of the artery. To restrict blood flow to the right half of the liver, the hepatic portal vein, bile duct, and the right hepatic artery should be tightened together with a catheter; the opposite arrangement is used for restricting blood flow to the left half of the liver, except that the left hepatic artery, instead of the right hepatic artery, is occluded[98]. It is important that the patient receive sufficient oxygen throughout the perioperative period, and that hepatotoxic drugs are avoided.

After hepatectomy, the patient should be closely monitored, with particular attention to abnormalities in levels of consciousness, liver function, the volume and character of drainage fluid, acid-base balance, and serum lactic acid levels. In general, during the first postoperative day, the ideal levels of serum hepatic transaminases, total bilirubin, and prothrombin activity can be expected to remain below 1000 IU/mL, about 2 mg/dL, and about 50%, respectively. Acidosis is very common in liver failure, so the level of serum lactic acid should be carefully monitored. Serum bilirubin level should rapidly decrease; if the level increases abruptly after the second postoperative day the risk of hepatic failure increases. Currently, there is not a unified definition of liver failure after hepatectomy. The international hepatic surgery research team has proposed a definition based on the normal postoperative course of serum bilirubin concentration and international normalized ratio (INR), reflecting the ability of the liver to maintain its synthetic, excretory, and detoxifying functions. Postoperative liver failure is defined as an increased INR and hyperbilirubinemia (according to the normal limits of the local laboratory) on or after postoperative day 5[104]. The severity of post-hepatectomy liver failure is graded based on its effect on clinical management; grade A failure requires no change in the patient's clinical management; grade B failure requires deviation from the usual management but does not require invasive therapy; grade C requires invasive treatment[104].

CONCLUSION

In conclusion, hepatectomy still has significant associated complications and mortality. These problems are closely related to surgical manipulations, anesthesia, preoperative evaluation and preparation, and postoperative observation and management. The safety profile of hepatectomy probably can be improved if the surgeons and medical staff involved have comprehensive knowledge of the expected complications and expertise in their management.

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MINIREVIEWS

Splanchnic-aortic inflammatory axis in experimental portal hypertension

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Author contributions: de las Heras N, Nava MP and Lahera V reviewed the cardiovascular physiology and pathology related to prehepatic portal hypertension; Regadera J reviewed the aortic pathology in experimental models of atherosclerosis; Aller MA and Arias J have integrated the knowledge about portal hypertension and inflammatory aortopathy in three progressive functional phases and wrote the final version of the manuscript.

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Abstract

Splanchnic and systemic low-grade inflammation has been proposed to be a consequence of long-term prehepatic portal hypertension. This experimental model causes minimal alternations in the liver, thus making a more selective study possible for the pathological changes characteristic of prehepatic portal hypertension. Low-grade splanchnic inflammation after long-term triple partial portal vein ligation could be associated with liver steatosis and portal hypertensive intestinal vasculopathy. In fact, we have previously shown that prehepatic portal hypertension in the rat induces liver steatosis and changes in lipid and carbohydrate metabolism similar to those produced in chronic inflammatory conditions described in metabolic syndrome in humans. Dysbiosis and bacterial translocation in this experimental model suggest the existence of a portal hypertensive intestinal microbiome implicated in both the splanchnic and systemic alterations related to prehepatic portal hypertension. Among the systemic impairments, aortopathy characterized by oxidative stress, increased levels of proinflammatory cytokines and profibrogenic mediators stand out. In this experimental model of long-term triple portal vein ligated-rats, the abdominal aortic proinflammatory response could be attributed to oxidative stress. Thus, the increased aortic reduced-nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase activity could be associated with reactive oxygen species production and promote aortic inflammation. Also, oxidative stress mediated by NAD(P)H oxidase has been associated with risk factors for inflammation and atherosclerosis. The splanchnic and systemic pathology that is produced in the long term after triple partial portal vein ligation in the rat reinforces the validity of this experimental model to study the chronic low-grade inflammatory response induced by prehepatic portal hypertension.

Key words: Portal hypertension; Inflammation; Aortopathy; Hepatic steatosis

Core tip: Triple partial portal vein ligation in the rat induces in the long term (22 mo) both splanchnic alterations, *i.e.*, liver steatosis and portal hypertensive intestinal vasculopathy associated with a portal hypertensive microbiome, and systemic alterations, *i.e.*, a wound-like inflammatory aortic response. These alterations support this experimental model of prehepatic portal hypertension for studying the pathophysiological mechanisms involved in the low-grade inflammatory response produced.

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INTRODUCTION

Portal hypertension is the most severe complication that develops in cirrhotic patients and is a leading cause of mortality worldwide[1]. Ascites, hepatorenal syndrome, life-threatening gastroesophageal bleeding, portosystemic encephalopathy and sepsis, derived from shunting of portal blood into the systemic circulation through neoformed collateral vessels, are the most serious and frequent clinical complications[1].

Portal hypertension is defined as an increase in portal blood pressure and is determined from the hepatic venous pressure gradient or pressure difference between the portal vein and the inferior vena cava[2]. The impairments arising from this pathological increase in portal pressure constitute the portal hypertensive syndrome[3].

EXPERIMENTAL PORTAL HYPERTENSIVE MODEL

The partial portal vein ligation experimental model in the rat is generally used to study portal hypertension since it has the lowest degree of hepatic impairment because portal hypertension is extrahepatic[4]. The surgical technique is simple and is based on making a calibrated stenosis of the portal vein[5]. If it is assumed that the intensity of the portal hypertension is determined by the resistance to the inflow produced by the constriction of the portal vein, this model of prehepatic portal hypertension could be improved by increasing the initial resistance to blood flow. With this objective in mind, we have modified this surgical technique by increasing the length of the stenosed portal tract with three equidistant calibrated stenosis[6].

This experimental model causes minimal alternations in the liver, thus making a more selective study possible for the pathological changes characteristic of prehepatic portal hypertension[4,6]. The experimental model of partial portal vein ligation is generally studied in the short-term, *i.e.*, 2-4 wk[4]. However, studying the late phases could be of great interest since the mechanisms involved and the related complications could be more similar to those found in chronic liver diseases in humans[1,2] which are related to the chronicity of portal hypertension, among other factors.

INFLAMMATORY RESPONSE RELATED TO PORTAL HYPERTENSION

Much evidence shows how inflammation contributes to the initiation and maintenance of portal hypertension[7,8]. Furthermore, early and chronic partial portal vein ligated-rats show hemodynamic and metabolic impairments, where the etiopathogeny is of an inflammatory nature[7,8]. Consequently, it could be hypothesized that chronic hemodynamic, vascular and metabolic changes in rats with prehepatic portal hypertension could have an inflammatory origin, most probably subsequent to splanchnic inflammation. In this way, the endothelial inflammatory mechanotransduction induced by portal hypertension could be the first step in the production of an inflammatory response in the intestinal wall. Thus, the early splanchnic endothelial disorder could induce, in turn, an inflammatory intestinal phenotype that would be linked to both phenomena: the gut microbiota alteration as well as the vasomotor impairments that are responsible for the splanchnic hyperdynamic circulation (Figure 1).

Furthermore, prehepatic portal hypertension induces the development of portal hypertensive enteropathy with inflammatory cell infiltration, particularly mast cells[9,10], which is reduced by the prophylactic administration of anti-inflammatory drugs, like budesonide and ketotifen[8,11]. Since the basic structural alteration found in portal hypertensive enteropathy is angiogenesis, the very appropriate name of "hypertensive portal intestinal vasculopathy" has been proposed[12].

The formation of new blood vessels could be a key mechanism in the pathogenesis of prehepatic portal hypertension[8]. Mast cells are involved in the regulation of physiological and pathological vasculogenesis by producing mediators, such as heparin, histamine, tryptase, transforming growth factor-β1, tumor necrosis factor-α (TNF-α), interleukins and cytokines, such as vascular endothelial growth factor[13]. The ability of mast cells to promote the synthesis and selective release of different angiogenic mediator molecules[14] would explain their participation in the splanchnic remodeling related to experimental prehepatic portal hypertension[8-10]. Lastly, intestinal mast cells are also a potent source of multiple chemokines and play an important role in immune regulation[15,16].

We have previously shown that prehepatic portal hypertension in the rat induces liver steatosis and causes changes in lipid and carbohydrate metabolism similar to those produced in chronic inflammatory conditions described in metabolic syndrome in humans[17-19]. Long-term portal hypertensive rats show a decrease in plasma adrenocorticotrophic hormone and corticosterone[20]. Glucocorticoids, such as cortisol and corticosterone, are pluripotent hormones that are vital in the host adaptation to stress. They are also essential for maintaining normal vascular tone, endothelial integrity and vascular permeability. Thus, the decrease in corticosterone in portal hypertensive rats could have deleterious effects on vascular systems. In addition, cortisol clearance is increased in individuals with fatty liver, and cortisol clearance is in turn inversely correlated with insulin sensitivity[21]. Therefore, in rats with portal hypertension, in which liver steatosis is present, a decreased stress responsiveness could be re-

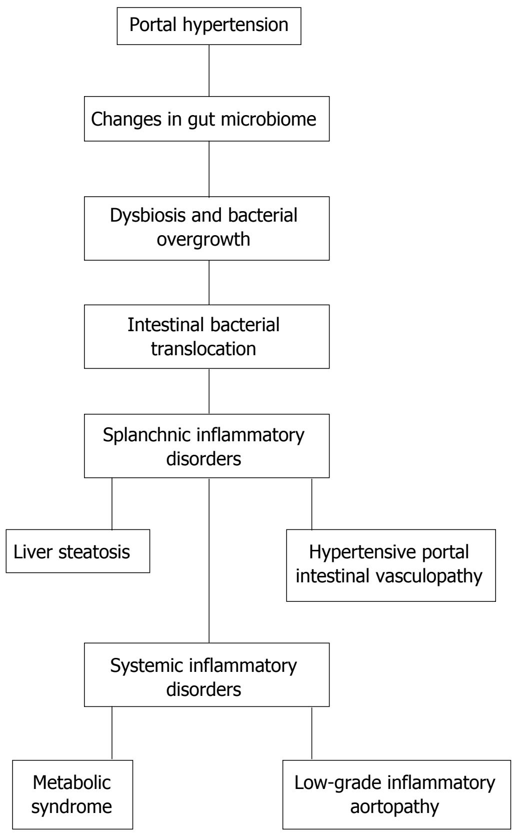

Figure 1 Splanchnic and systemic alterations in rats with prehepatic portal hypertension produced by triple partial portal vein stenosis.

lated to an impaired metabolic feedback system. The decreased neuroendocrine response to stress and systemic chronic inflammation would be another link between disordered lipid metabolism and inflammation in the evolution of this experimental model[22]. So, rats with long-term portal hypertension presenting systemic low-grade inflammation and decreased responsiveness to stress could inappropriately switch carbohydrate metabolism to predominant lipid metabolism, thus inducing body energy imbalance and ultimately, hepatic steatosis and metabolic syndrome[18,19]. Consequently, data from animal models proved that mast cells are directly involved in diet-induced obesity, diabetes and metabolic syndrome[23].

The association of liver steatosis and metabolic syndrome with inflammation is well documented[24]. Hence, we could hypothesize that inflammation, probably of splanchnic origin, could be the pathophysiologic link between the metabolic syndrome with liver steatosis and aortic vascular disease in portal hypertensive rats. The association between metabolic and atherogenic alterations with a proinflammatory aortic phenotype could suggests the possibility that portal hypertension might constitute a novel risk factor for cardiovascular disease[20,25] (Figure 1).

PORTAL HYPERTENSIVE INTESTINAL MICROBIOME

Bacterial intestinal translocation occurs in acute portal hypertension[26] and in chronic portal hypertension in the adult partial portal vein ligated rat[27] when there are associated precipitating factors, such as hemorrhagic shock[28]. However, in chronic (1 mo) prehepatic portal hypertension by triple partial portal vein ligation, there are gut microflora alterations with less positive cultures of *Enterococci* and lactic acid bacteria, associated with bacterial translocation to the mesenteric lymph nodes[29]. Bacterial overgrowth in the intestinal tract may be the most important factor in bacterial translocation, in particular when associated with splanchnic inflammation and increased intestinal permeability, secondary to portal hypertension[29]. It has also been proposed that bacterial translocation may render the gut a "cytokine-releasing" organ that, at the same time, would induce nitric oxide overproduction and the development of the hyperdynamic circulatory state. At the same time, this is one of the progressive characteristics of portal hypertension[30]. Bacterial overgrowth could also be caused by delayed transit, mucosal hypoperfusion and oxidative damage, which increases intestinal permeability and induces the transmural passage of bacteria in portal hypertensive rats[31,32].

Although microbial communities reside on all mammal body surfaces, including the skin and respiratory, gastrointestinal and genitourinary tracts, the largest collection of microbes reside in the gut[33]. The intestinal microbiome is considered more than just a simple organ of the mammal body. Cooperative interactions between intestinal microbes and their hosts typically involve microbial participation in host functions such as defense, metabolism, and reproduction[34]. However, communications between the host and its gut microbiota are altered in pathophysiological processes, especially if associated with inflammation, including portal hypertension[35]. Diseases mediated by the inflammatory response could induce a change in the relationship of the rat body with gut microbiota, the significance of which is unknown[35].

Inflammatory conditions, such as splanchnic inflammation related to portal hypertension, could induce a change in the mammalian organism and gut microbiota relationship. In particular, splanchnic inflammation not only could alter gut microbiota composition, but also cause epithelial and endothelial permeability of the intestinal bacteria to increase bacterial products, such as toxins[34]. Bacterial translocation secondary to portal hypertension could lead to bacterial overgrowth and disruption of gut homeostasis[29]. This results in a "leaky gut" syndrome, with translocation of gut bacteria and bacterial products, also called pathogen-associated molecular patterns or PAMPs, to systemic sites, that finally results in systemic complications[34-37].

Gut microbiota in rats with portal hypertension could contribute to the development of liver steatosis and metabolic syndrome. The gut microbiota may be involved in hepatologic conditions including non-alcoholic fatty liver disease[33]. Thus, bacterial products, including endotoxins, can affect Kupffer cells, hepatocytes and hepatic stellate cells, which participate in the initiation and progression of non-alcoholic fatty liver disease[38]. Gut microbiota

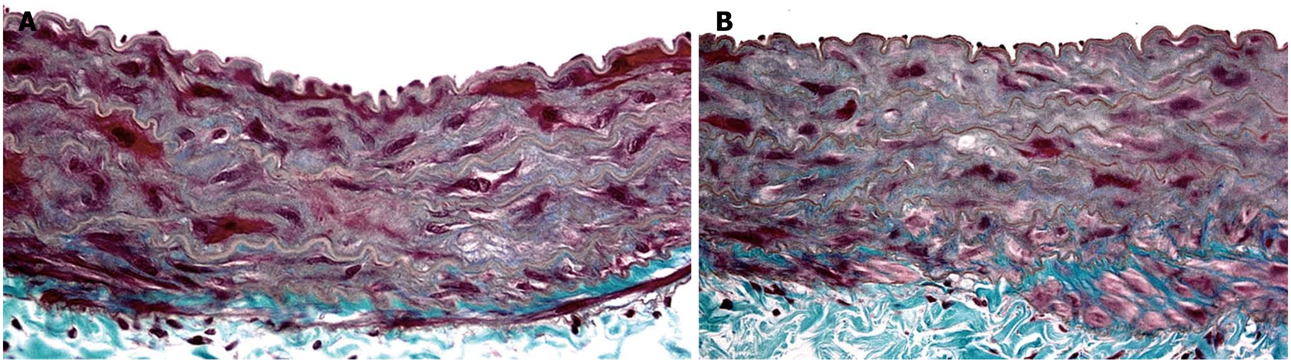

Figure 2 Aortic abdominal wall microscopic images in long-term (22 mo) sham-operated rat (A) and in a triple partial portal vein ligated-rat (B). The aortic wall in the rat with portal hypertension is enlarged, has more fibrosis, with collagen deposition in the middle layer, a greater loss of the smooth muscular cell nucleus and much thinner elastic fibers than in the aortic wall of the sham-operated rat. They are also are distributed in an irregular manner (Masson, × 40).

could also contribute to the development of hypertensive portal intestinal vasculopathy. Recent results suggest that the increased intestinal vascularization is mediated through microbiota-induced angiopoietin-1 expression in the intestinal epithelium[39].

Gut microbiome responses to portal hypertension could be a central event in the pathogenesis of splanchnic, *i.e.*, liver steatosis and enteropathy, and systemic inflammatory conditions. However, it has been also suggested that microbiome changes could alter host-microbiome interactions to mitigate disease[33] (Figure 1).

WOUND-LIKE INFLAMMATORY AORTIC RESPONSE

Long-term experimental prehepatic portal hypertension represents a risk factor of an inflammatory nature for aortic disease development[20,25]. Triple partial portal vein ligation in the rat induces an abdominal aortic inflammatory response 22 mo after the operation. These portal hypertensive rats show significant histological changes, in particular in the middle layer of the aortic wall. The elastic fibers lose their orderly circumferential arrangement. The interstitial connective tissue is enlarged with fibrosis and associated with a dramatic decrease in the number of smooth muscle cells. Finally, immature collagen also increases with the degeneration of connective tissue[20] (Figure 2).

In this experimental model of long-term triple portal vein ligated rats, the abdominal aortic proinflammatory response could be attributed to oxidative stress. In this way, the increased aortic reduced-nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase activity[20] could be associated with reactive oxygen species production and promote aortic inflammation[40]. Also oxidative stress mediated by NAD(P)H oxidase has been associated with risks factors for inflammation and atherosclerosis[41].

In chronic portal hypertensive rats, over-activation of endothelial nitric oxide synthase (eNOS) might cause aortic nitric oxide overproduction[20,25]. Upregulation of eNOS has also been seen in the aorta of short-term portal vein stenosed and biliary cirrhotic rats, respectively[42]. It has been suggested that an increased basal release of nitric oxide has a major role in the pathogenesis of vasodilation and vascular hypocontractility associated with portal hypertension[7]. Lipopolysaccharide (LPS) administration to cirrhotic rats increases aortic eNOS activation but, on the contrary, decreases eNOS protein expression and activity in superior mesenteric arteries. These results may explain the worsening of the hyperdynamic state in cirrhosis during septic shock by direct LPS-induced eNOS activation in large systemic vessels, and its inhibition in concomitant small splanchnic vasculature[43].

The essential role of reactive oxygen species in the chronic inflammatory response has led to the view that reactive oxygen species promote inflammation[44]. Reactive oxygen species can increase the expression of inducible genes leading to the synthesis of cytokines, chemokines, chemokine receptors and adhesion molecules. These actions rely on transcription factors, such as nuclear factor κB (NF-κB)[44]. Aortic overproduction of proinflammatory cytokines, including TNF-α, interleukin-1β (IL-1β), and IL-6 in chronic portal hypertensive rats, associated with an increased NF-κB/NF-κB inhibitor (IκB) ratio supports the existence of a proinflammatory abdominal aortic response. If so, reactive oxygen species or TNF-α could induce activation of the I$\kappa\kappa$ (IκB kinase) complex resulting in phosphorylation of IκB, subsequent translocation of NF-κB to the nucleus and expression of NF-κB responsive genes (Figure 3).

Proinflammatory cytokines, cytokine-dependent pathways and immune cells have been implicated in the development of cardiovascular diseases, *i.e.*, atherosclerosis, coronary artery disease, chronic heart failure and hypertension[44]. Thus, a large body of evidence supports the involvement of common proinflammatory cytokines in the development and progression of a systemic low-grade inflammation affecting the cardiovascular system. In addition, these low-grade inflammatory cardiovascular diseases can be aggravated when a new inflammatory process, either of infectious origin or autoimmune nature, is added[45,46]. This is the reason why it could also be considered that prehepatic portal hypertension produces

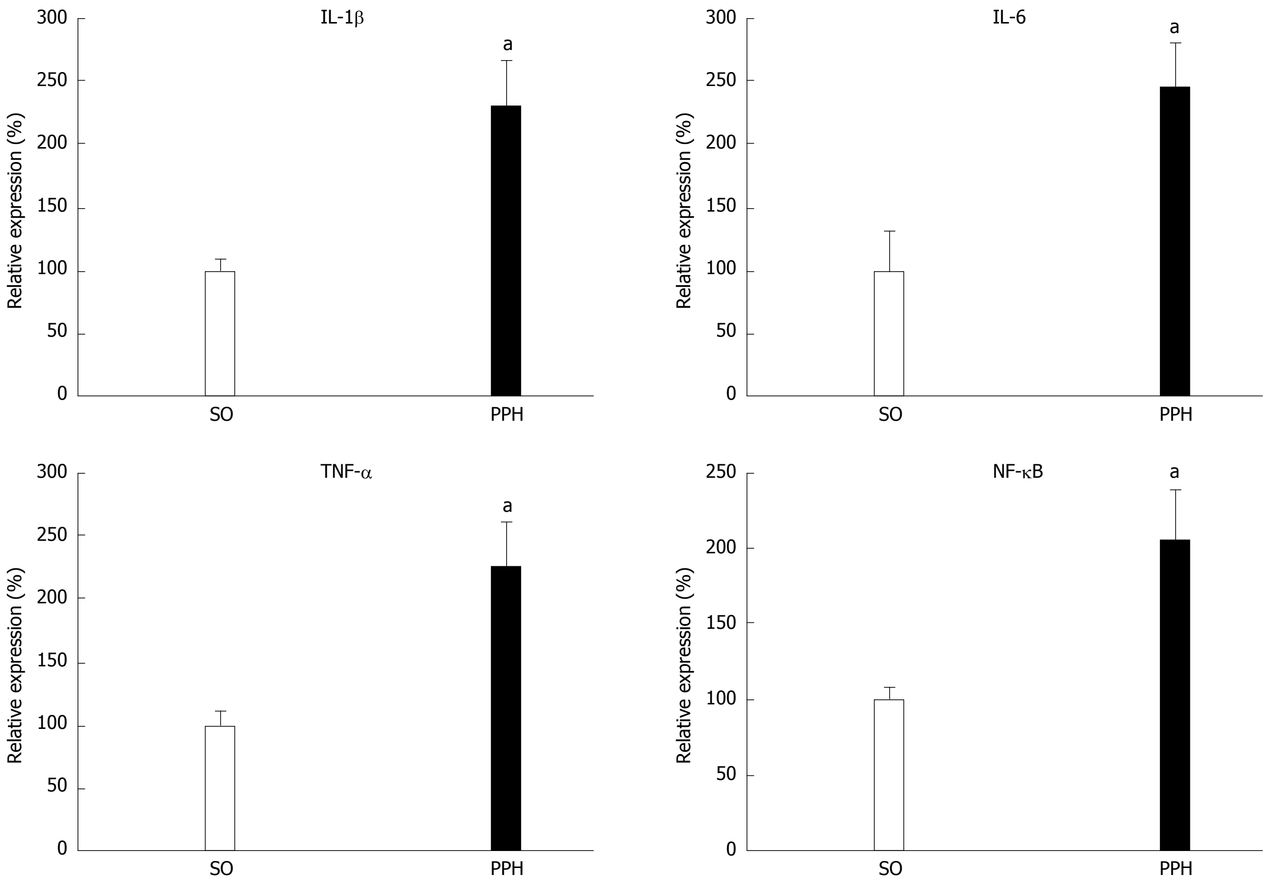

Figure 3 Aortic inflammatory mediators in triple partial vein ligated-rats at 22 mo after postoperative evolution. Increased aortic mRNA expression of tumoral necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, are associated with the increased expression of mRNA levels of the nuclear factor κB (NF-κB)/NF-κB inhibitor (IκB) ratio. SO: Sham-operated rats; PPH: Prehepatic portal hypertensive rats. ^{a}P < 0.05, statistically significant value in regards to SO-rats.

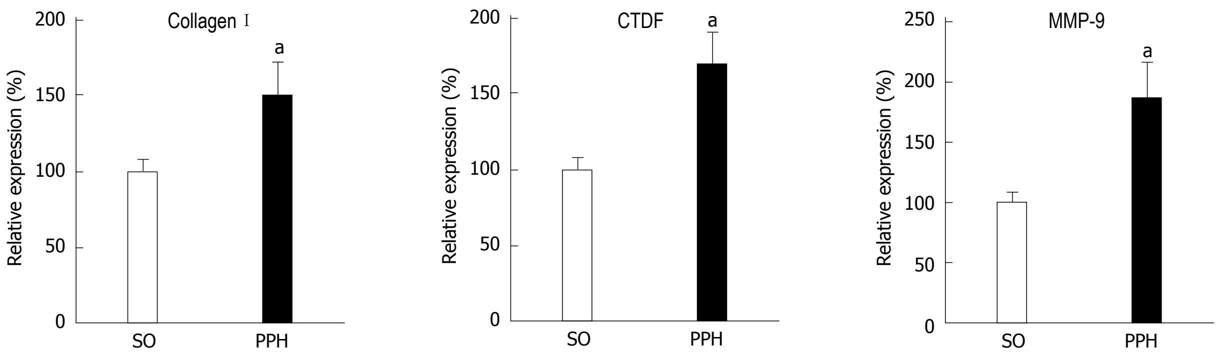

Figure 4 Aortic profibrogenic mediators in triple partial vein ligated-rats at 22 mo of postoperative evolution. Increased abdominal aortic expression of matrix metalloproteinase (MMP)-9, collagen Ⅰ and connective tissue growth factor (CTGF). SO: Sham-operated rats. PHH: Prehepatic portal hypertensive-rats. ^{a}P < 0.05, statistically significant value in regards to SO-rats.

a low degree inflammatory cardiovascular response, which could be aggravated when a new inflammatory process (acute-over-chronic) is added, particularly infections or hepatic insufficiency[8].

Mast cells stand out among the potential mediators of the low-grade inflammatory response supposedly involved in metabolic and vascular diseases in the experimental model of prehepatic portal hypertension[3,9]. Nonetheless, these results obtained in the short-term evolution of portal hypertensive rats cannot be extrapolated to long-term portal hypertensive rats. Thus, a study of the role of the splanchnic subpopulations of mast cells in chronic portal hypertensive rats would be interesting, given that the gut, particularly with impaired intestinal barrier function, plays an important pathophysiological role in chronic inflammation in cardiovascular diseases[47]. In particular, mast cells play a key role in experimental atherosclerosis and can modulate the inflammatory aortic response through numerous proinflammatory mediators, including TNF-α, IL-6 and metalloproteinases[48,49]. In addition, mast cell chymase also functions as an angiotensin-converting enzyme, particularly in rodents and there-

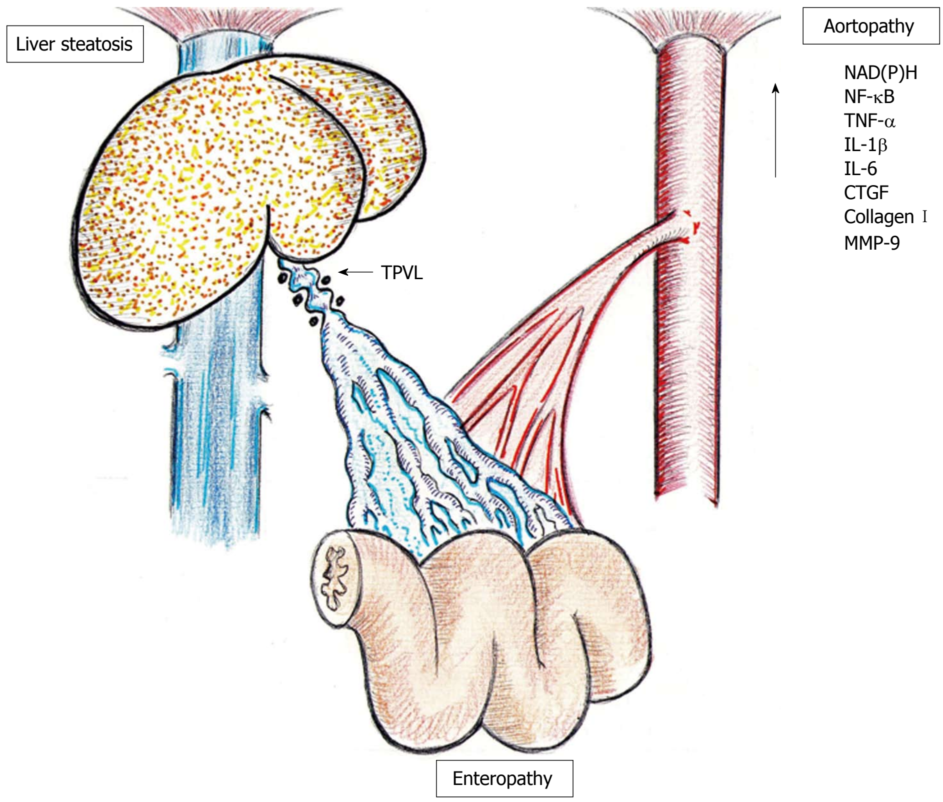

Figure 5 Chronic splanchnic alterations, liver steatosis and enteropathy, secondary to long-term triple partial portal vein stenosed rats are associated with oxidative stress, inflammatory cytokines and profibrogenic mediators in abdominal aorta. NAD(P)H: Reduced-nicotinamide-adenine dinucleotide phosphate; MMP: Matrix metalloproteinases; TPVL: Triple partial portal vein stenosed; NF-κB: Nuclear factor κB; TNF-α: Tumor necrosis factor-α; IL: Interleukin; CTGF: connective tissue growth factor; MMP-9: Matrix metallopeptidase 9.

fore contributes to aortic fibrosis[50] (Figure 4).

Lastly, enhanced aortic mRNA expression of oxidative and inflammatory mediators are associated with increased aortic expression of collagen I and connective tissue growth factor (CTGF) in long-term portal hypertensive rats[20,25]. Increased aortic CTGF expression could regulate aorta collagen remodeling, and therefore enhance the synthesis of extracellular matrix proteins, particularly type I collagen. In turn, CTGF could activate the NF-κB pathway and increase proinflammatory gene expression[51]. These results suggest the existence of a proinflammatory and a profibrotic aortic phenotype in long-term prehepatic portal hypertensive rats (Figures 4 and 5).

CONCLUSION

In summary, rats with long-term portal hypertension with liver steatosis and hypertensive portal intestinal vasculopathy also suffer a low-grade abdominal aortic inflammation associated with fibrosis.

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ORIGINAL ARTICLE

Identification and characterization of a novel bipartite nuclear localization signal in the hepatitis B virus polymerase

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Abstract

AIM: To characterize the nuclear import of hepatitis B virus (HBV) polymerase (P) and its relevance for the viral life cycle.

METHODS: Sequence analysis was performed to predict functional motives within P. Phosphorylation of P was analyzed by in vitro phosphorylation. Phosphorylation site and nuclear localization signal (NLS) were destroyed by site directed mutagenesis. Functionality of the identified NLS was analyzed by confocal fluorescence microscopy and characterizing the karyopherin binding. Relevance of the structural motives for viral life cycle was studied by infection of primary *Tupaia* hepatocytes with HBV.

RESULTS: We identified by sequence alignment and functional experiments a conserved bipartite NLS containing a casein kinase Ⅱ (CKⅡ) phosphorylation site located within the terminal protein domain (TP) of the HBV polymerase. Inhibition of CKⅡ impairs the functionality of this NLS and thereby prevents the nuclear import of the polymerase. Binding of the import factor karyopherin-α2 to the polymerase depends on its CKⅡ-mediated phosphorylation of the bipartite NLS. In HBV-infected primary *Tupaia* hepatocytes CKⅡ inhibition in the early phase (post entry phase) of the infection process prevents the establishment of the infection.

CONCLUSION: Based on these data it is suggested that during HBV infection the final import of the genome complex into the nucleus is mediated by a novel bipartite NLS localized in the TP domain of HBV polymerase.

Key words: Hepatitis B virus; Nuclear localization signal; Casein kinase Ⅱ; Trafficking; Replication

Core tip: The mechanism mediating import of the hepatitis B virus (HBV) genome into the nucleus is still not fully understood. We describe the identification and characterization of a bipartite nuclear localization signal (NLS) in the HBV polymerase that harbours a phosphorylation site for casein kinase Ⅱ (CKⅡ). Integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CKⅡ prevents karyopherin-α2 from binding to the polymerase and thereby the import of the polymerase is impaired. Analysing the viral life cycle we observed that inhibition of CKⅡ blocks the import of the genome into the nucleus resulting in impaired cccDNA formation and so the establishment of the viral infection is prevented.

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INTRODUCTION

Infection with human hepatitis B virus (HBV) can cause acute or chronic inflammation of the liver. At present there are about 400 million chronically infected people worldwide. Moreover, persistently infected individuals have an increased risk of developing primary hepatocellular carcinoma (HCC)[1-6]. HBV is the prototype member of the hepadnaviridae family, which encompasses members isolated from woodchucks, ground squirrels and avian viruses isolated from *e.g.*, pekin duck, grey heron and stork.

The HBV polymerase (P) has four domains (Figure 1A). The terminal protein domain (TP) contains the tyrosine residue that primes DNA synthesis and covalently links P to the viral DNA[7-9]. The spacer domain has no known function other than to connect the terminal protein domain with the rest of P. The spacer domain, however, harbors at aa position 320 a thrombin cleavage site. This generates the possibility that not a full length protein, but a truncated polymerase is linked to the HBV genome due to intracellular proteolytic processing[10]. The reverse transcriptase domain and the RNase H domain contain the two enzymatic active sites catalyzing the reverse transcription of the RNA template to DNA and degradation of the RNA template.

Regarding the life cycle of HBV one open question concerns the fate of the viral nucleocapsid after the virus has entered the cell. There is evidence that the virus enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid is released into the cytoplasm[11,12] and transported towards the nuclear pore complex[13,14]. Productive viral infection requires the transport of the HBV genome into the nucleus where the conversion into cccDNA occurs[15].

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex[16-18] where a partial disassembly of the DNA-loaded nucleocapsid leads to a release of the polymerase-linked genome[13,19-21]. The polymerase-genome complex is too big for free diffusion through the nuclear pore complex.

Due to the facts that that although P is too big for free diffusion through the nuclear pore complex the polymerase-genome complex is imported into the nucleus and that a fraction of P protein is found within the nucleus[22-24] we examined the P protein for conserved motifs that could play a role for nuclear import and aimed to characterize the nuclear import of HBV polymerase-genome complex.

MATERIALS AND METHODS

Cell lines and culture conditions

The human hepatoblastoma cell lines HuH-7[25] and HBV producing cell line HepG2.2.15[26] were cultured in D-MEM medium containing 10% (v/v) fetal calf serum (FCS), 500 U/L penicillin and 100 mg/L streptomycin (PAA, Pasching, Austria). Inducible HBV producing cell line HepAD38[27] were cultivated like HepG2.2.15 but with 400 mg/L G418, 50 μmol/L hydrocortisone and 2.5 mg/L insulin (Sigma-Aldrich, Sleeze, Germany), additionally.

Subcellular fractionation

Subcellular fractionation was performed as described[28,29].

Infection of primary hepatocytes

Primary *Tupaia belangeri* hepatocytes were isolated, cultivated and infected as described[30,31]. Trypsin treatment for removal of attached viral particles was performed as described[12,31-33]. HBeAg and HBsAg synthesis were analysed 120 h after infection.

Generation of expression constructs

Plasmids were sub-cloned in *Escherichia coli* strain DH5α. The relevant mutations in the listed primer sequences are highlighted, restriction sites underlined and the corresponding backward primer sequences of mutation primers are reverse complementary to the forward primer if not citied otherwise.

The 1.2 fold HBV genome pJO19 (subtype ayw, genotype D) was derived from plasmid pSM2[26] by a stepwise truncation of the plasmid with *Bse*A Ⅰ and *Aat* Ⅱ. Mutant versions of the wild type genome with alterations in the polymerase coding sequence were generated based on pJO19: The CKⅡ recognition site deficient genome pJO19[T100I] was generated using primer ΔCKⅡ_fw (5'-CAg TTT gTA ggC CCA CTC ATA gTT AAT gAg AAA AgA AgA TTg CAA TTA ATT ATg CCT gCC), the pseudophosphorylated genome pJO19[T100D] was generated with primer *CKⅡ_fw (5'-CAg TTT gTA ggC CCA CTCg ACg TTA ATg AgA AAA gAA gAT TgC AAT TAA TTA TgC CTg CC), the genome with an inactivated NLS pJO19[K105D,K106S] was generated with forward primer ΔNLS_fw (5'-ggC CCA CTC ACA gTT AAT gAg CAg TCT AgA TTg CAA TTg ATT ATg CCT g). GFP wild type expression was obtained by pEGFP-N1 (BD, Heidelberg, Germany). The N-terminal fusions of NLS signals to GFP were generated from a modified version of pEGFP-N1 (pJO21). In pJO21 the first base of the transcriptional START of the GFP reading frame was deleted by site directed mutagenesis to prevent wild type GFP expression using primer GFP Δstart_fw (5'-CCA CCg gTC gCC ACC Tgg TgA gCA

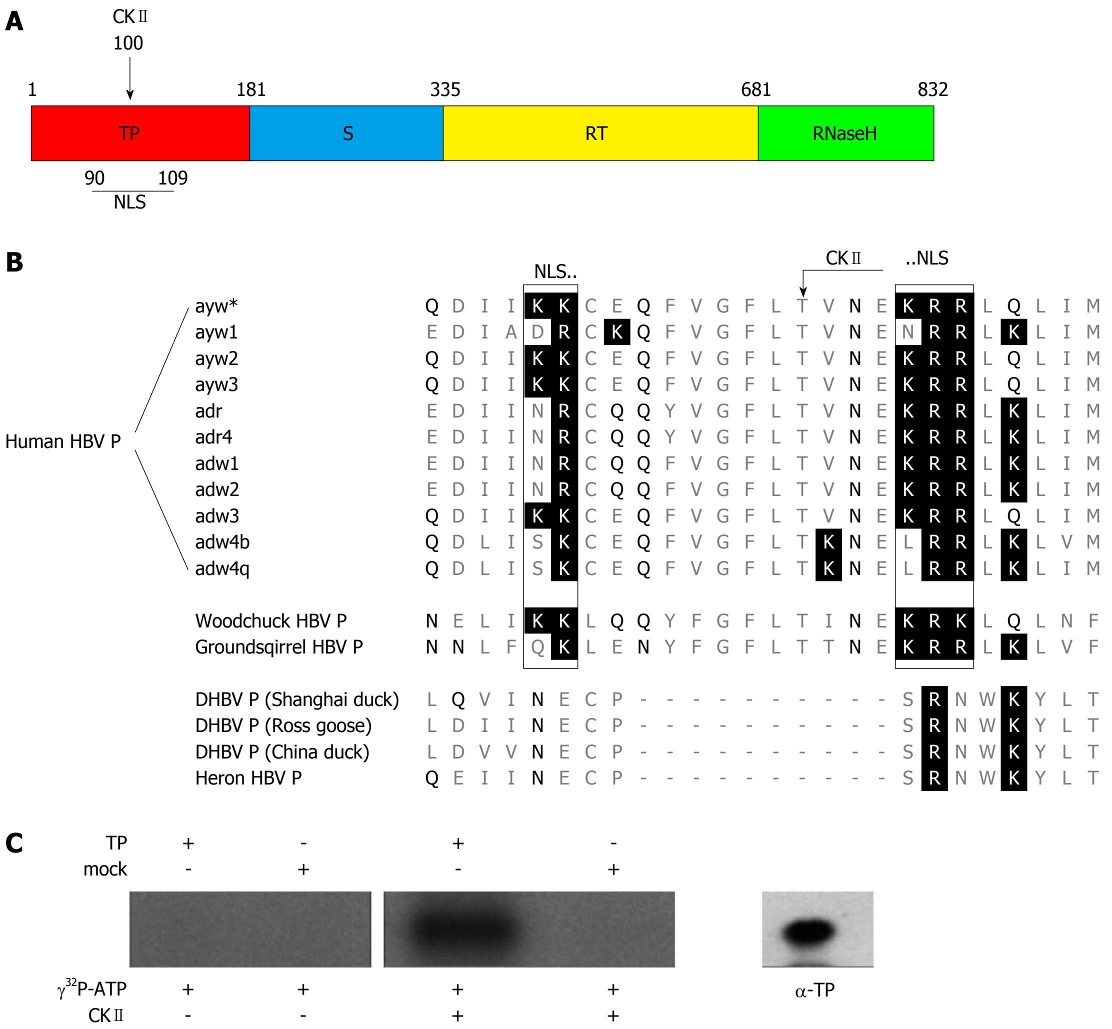

Figure 1 Sequence alignment of the hepatitis B virus polymerase from various virus subtypes and species. A: Scheme of the hepatitis B virus (HBV) polymerase showing the different domains. The terminal protein domain (TP) is shown in red, the spacer domain (S) in blue, the reverse transcriptase domain (RT) in yellow and the RNase H domain in green. The numbers designate the amino acids referred to HBV genotype D. The positions of the casein kinase Ⅱ (CKⅡ) phosphorylation site and of the bipartite nuclear localization signal (NLS) identified in this study (see Figure 1B) are indicated; B: This figure shows amino acid alignment of Q86-M111 referred to the sequence of subtype ayw*, which was used in this study. Basic amino acids are highlighted by a black background and polar (δ+) amino acids are highlighted by black letters. A conserved protein kinase CKⅡ recognition site (arrow) was identified in orthohepadnaviruses at Thr100 (protein kinase CK Ⅱ: T/S-X-X-E/D; X = any amino acid). A bipartite nuclear localization signal was identified in *orthohepadnaviruses*, which is flanking the CKⅡ recognition site by its two basic amino acid clusters (rectangles). All identified putative motifs were not found in the aligned P proteins of the compared *avihepadnaviruses*; C: Purified TP domain and mock purified proteins (empty vector products) were incubated with [γ-^{32}P] ATP and recombinant protein kinase CKⅡ. To control auto-phosphorylation TP domain was incubated with [γ-^{32}P] ATP in absence of the kinase. The protein specificity was verified by Western blotting using a TP specific antibody (α-TP) on a separate lane. All experiments were performed in triplicate. One representative is shown.

Agg gCg Agg). The plasmid pNLS*NP*-GFP was generated by amplifying human nucleoplasmin from a cDNA library. The polymerase chain reaction (PCR) product was flanked by terminal *Bgl*Ⅱ sites, which were generated by primer N-NLS-3fw (5'-TTT AgA TCT gTT CAg ggC CAg TgC) and primer N-NLS-3bw (5'-TTT AgA TCT TTT TAC TTT TTT CTg Tgg). The PCR product was ligated to the *Bam*HI cut pJO21. An engineered *Bgl*Ⅱ site followed by an optimized Kozak sequence[34] with transcriptional START was inserted immediately upstream of the NLS sequence by site directed mutagenesis using forward primer N-NLS-4fw (5'-ggA TgT gAA ACT CTT AAg TAg ATC TCg CCA CCA Tgg gAA AgC ggT CTg CCC CTg g). The dispensable upstream sequence was removed by a *Bgl*Ⅱ digest. Analogue to pNLS*NP*-GFP, the plasmid pNLS*TP*-GFP was generated by amplifying the putative NLS of the TP domain from pJO19 using forward primer TP-NLS-3fw (5'-CCC ggA TCC ATg CCC CTA TCC TAT CAA CAC) and backward primer TP-NLS-3bw (5'-TTT AgA TCT TCT TTT CTC ATT AAC Tg). The upstream *Bgl*Ⅱ site, the Kozak signal and the transcriptional START was inserted using primer TP-NLS-4fw (5'-CCT AAT ATA CAT TTA CAC CAA gAC AgA TCT CgC CAC CAT ggT gAA AAA ATg TgA ACA gTT TgT Agg C).

The P-expression constructs were generated based on pJo19 or the corresponding mutants by PCR and subcloned in the pCDNA.3 eukaryotic expression vector.

Site-directed mutagenesis

was performed as described[35] by amplification of the whole plasmid using *Pfu* Turbo Hotstart DNA-Poly-

merase (Invitrogen, Karlsruhe, Germany). All synthetic oligonucleotides are purchased by Tib-Molbiol, Berlin, Germany.

Purification of recombinant proteins

The coding sequence for the TP domain (amino acid 1-181) of HBV polymerase was amplified by PCR and inserted into the eubacterial expression vector pQE60 (Qiagen, Hilden, Germany), which encodes a C-terminal His-tag. Expression was performed at room temperature to reduce the formation of inclusion bodies. The soluble fraction of recombinant TP was purified by affinity chromatography on a Ni-NTA column under native conditions as described recently[36]. TP protein inclusion bodies were solved using 6 mol/L guanidine hydrochloride. Ni-NTA affinity purification under denaturing conditions was performed as described[37]. For further purification the TP containing fractions were pooled, dialyzed to buffer A_{MS} (6 mol/L urea, 20 mmol/L sodium acetate, 2% (v/v) ethanol, pH 5.5) and polished by cationic exchange chromatography using a pre-packed Tricorn MonoS column (GE Healthcare, Freiburg, Germany). The elution was performed by a linear gradient over 20 column volumes (cv) between buffer A_{MS} and A_{MS} containing 1 mol/L sodium chloride.

In vitro phosphorylation

experiments were performed using highly purified *E. coli* produced terminal protein domain dialyzed against kinase buffer (25 mmol/L Tris-HCl, 25 mmol/L beta-glycerophosphate, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, pH 7.5). Phosphorylation was started by addition of 10 μCi [γ-^{32}P] ATP and recombinant human CKⅡ (Merck, Darmstadt, Germany). After 30 min incubation at 30 ℃ the reaction was stopped by addition of SDS sample buffer and heat treatment (5 min, 95 ℃). Proteins were separated by 12% (v/v) SDS-PAGE and detected by autoradiography.

On column phosphorylation of was performed using polished, denatured TP from the cationic exchange chromatography. After addition of 20 mmol/L 2-mercaptoethanol and 100 mmol/L Tris, pH 8 the TP containing fraction was incubated for 1 h at room temperature with 2 cv Ni-NTA agarose, which was pre-washed with buffer A_D (6 mol/L urea, 100 mmol/L Tris, pH 8.0). The coupling efficiency was 90%, which was determined by optical density at 280 nm. Equal amounts of TP-agarose were loaded on two empty chromatography columns. A controlled refolding of TP was initiated by a 30 cv linear gradient of buffer A_D to buffer R (20 mmol/L Tris, 134 mmol/L sodium chloride, 10% (v/v) glycerol, 10% (v/v) sucrose, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). The buffer was changed by a 10 cv linear gradient to buffer K (20 mmol/L Tris, 50 mmol/L potassium chloride, 10 mmol/L imidazole, 20 mmol/L 2-mercaptoethanol, 20 mmol/L beta-glycerol phosphate, 0.1 mmol/L sodium ortho-vanadate, 0.1% (v/v) Tween-20, pH 7.5). 2500 U recombinant protein kinase CKⅡ (Merck, Darmstadt, Germany) was injected together with 200 μmol/L GTP in buffer K and the column was incubated for 3 h at 28 ℃. The reaction was stopped by washing the column with 5 cv buffer K.

Binding partner fishing

Six confluent grown 175 cm² culture flasks of HuH-7 cells were lysed by sonification in TBS buffer including protease inhibitor cocktail (1 mmol/L PMSF, 5 mg/L aprotinin, 1 mg/L pepstatin, 4 mmol/L leupeptin, 1 mmol/L EDTA). The crude lysate was cleared by centrifugation at 20000 rpm in a TST41 rotor. The lipid content of the supernatant was reduced by precipitation of the proteins at 75% (w/v) ammonium sulfate. The protein pellet was resolved in TBS buffer and desalted by gel filtration using a HiTrap Desalting column (GE Healthcare, Freiburg, Germany). The desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate was diluted in buffer B (20 mmol/L Tris, 25 mmol/L beta-glycerol phosphate, 1 mmol/L ortho-vanadate, 20 mmol/L 2-mercaptoethanol, 0.1% (v/v) Tween-20, pH 7.5). Equal amounts of this protein solution were injected to the two terminal protein bound columns and to a blank column only loaded with nickel-agarose. After washing the three columns for 5 cv with buffer B the binding partners were eluted by 1 mol/L sodium chloride and analyzed by western blotting using antibody karyopherin-α2 (C-20) purchased from Santa Cruz, Heidelberg, Germany.

Kinase inhibitor experiments

HepG2.2.15 cells were seeded in 6-well plates with an initial density of 5×10^5 cells/well. Three days after cell seeding CKⅡ inhibitor DMAT (Merck, Darmstadt, Germany) was added to the consumed cell culture medium for 2.5 h. After washing with phosphate buffered saline the cells were incubated for additional 18 h with consumed cell culture medium from the non-HBV producing cell line HuH-7, supplemented with the same concentration of DMAT as already pre-treated. The concentration of the solvent dimethyl sulfoxide (DMSO) was kept at 0.7% (v/v) in all investigated samples.

HBV quantification

Virus genomes were extracted from cell culture supernatant using High Pure Viral Nucleic Acid Kit and determined by LightCyler PCR (Roche, Mannheim, Germany) using a HBx specific probe[38]. cccDNA was extracted from HBV infected *Tupaia* hepatocytes according standard protocols for genomic DNA extraction with phenol/chloroform[39]. Southern blotting of HBV DNA using a HBV specific ^{32}P labeled probe was performed as described[31].

Endogenous polymerase reaction

HuH-7 cells (3×10^5) were seeded in 6-well plates and transfected with 2 μg HBV DNA using Fugene 6 (Roche, Mannheim, Germany). The enveloped viral particles were precipitated 5 d after transfection by sheep anti-HBs polyclonal serum (kindly gift from Klaus-H. Heermann, University Goettingen, Dept. Virology, Germany) and swollen protein-A sepharose beads (Sigma-Aldrich,

Sleeze, Germany) from the cell culture supernatant. The endogenous polymerase reaction (EPR) reaction was performed as described[40] using 10 μCi [α-^{32}P] dCTP (GE Healthcare, Freiburg, Germany) for the labeling.

Confocal laser scanning microscopy

HuH-7 cells (5 × 10^4) were grown on cover slides in 24-well plates and fixed with 4% formaldehyde/PBS for 30 min at 25 ℃. For visualization of actin filaments, the cells were stained with FITC-labelled phalloidin (Sigma, Munich, Germany). Staining was performed as described[41,42]. Rabbit-derived polyclonal TP-specific or spacer domain-specific sera were used for detection of P. Confocal laser scanning microscopy (CLSM) immunofluorescence was performed using the Zeiss LSM 510 microscope (Zeiss, 20 × and 63 × objectives).

RESULTS

Identification of conserved motifs on HBV polymerase

Previous data of our lab based on cell permeable HBV capsids[13] and studies by M Kann's lab[21] argue against the concept that intact viral capsids[17] or HBcAg shuttles the genome-polymerase complex into the nucleus[16,20]. Based on the data from the TLM-nucleocapsid model system it can be assumed that a partial disassembly of the capsid occurs within the nuclear pore complex or in a perinuclear domain that leads to a release of the genome complex. This raises the question about the final import of the polymerase linked genome into the nucleus.

Sequence analysis of HBV polymerase subtype ayw predicted the existence of a bipartite nuclear localization signal within the terminal domain (TP) (amino acid K90-K91, K104-R106) (Figure 1A). Moreover, a phosphorylation site for CKⅡ (T100) was found within the putative NLS (Figure 1B). The family hepadnaviridae encompasses two genera: orthohepadnavirus and avihepadnavirus. Further analysis revealed that the bipartite NLS and the enclosed CKⅡ phosphorylation site are conserved within the *orthohepadna* viruses but not within the *avihepadna* viruses (Figure 1C). The existence of a functional NLS would enable the transfer of the genome-polymerase complex through the nuclear pore complex into the nucleus.

TP domain is phosphorylated by CKⅡ *in vitro*

To control experimentally whether the predicted phosphorylation site indeed can be phosphorylated by CKⅡ *in vitro* phosphorylation was performed. Thereto, highly purified recombinant TP domain was incubated with [γ-^{32}P] ATP in the presence of CKⅡ. To exclude any phosphorylation by contaminating kinases the purified TP domain was incubated as described above, but CKⅡ was omitted. As an additional control a mutated TP domain was used in which the predicted CKⅡ phosphorylation site was destroyed by a T to A conversion at aa position 100. Figure 1B shows a significant specific phosphorylation of the TP domain only if CKⅡ is present. In case of the controls no significant phosphorylation was observed. To confirm the identity of the phosphorylated species with the TP domain Western blotting analysis was performed (Figure 1C, right panel). This indicates that the predicted kinase recognition site on the terminal protein is indeed accessible for phosphorylation.

P protein harbors a functional bipartite NLS, which depends on phosphorylation

To study the functionality of the TP-derived putative bipartite NLS HuH-7 cells were transfected with an expression plasmid encoding for a fusion protein of the putative NLS and GFP (NLS*P*-GFP). As a positive control served a 17 aa long prototype NLS (K142 to K158) derived from human nucleoplasmin (gi114762) fused to the amino terminus of GFP (NLS*NP*-GFP). The intracellular distribution of the GFP fluorescence was quantified by confocal laser scan microscopy in living cells. Compared to wild type GFP expression, which was found evenly distributed within the cell (Figure 2A), the level of NLS*P*-GFP was approximately 30% higher in the nucleus then in the cytosol (Figure 2B). In case of the positive control (NLS*NP*-GFP) an about 75% elevated level of GFP specific fluorescence in the nucleus was observed (Figure 2C). This confirms that the predicted sequence indeed acts as a functional NLS. To analyze a putative relevance of CKⅡ-dependent phosphorylation for the functionality of the TP-derived NLS, NLS*P*-GFP producing cells were incubated for 2 h with CKⅡ inhibitor DMAT prior analysis by confocal microscopy. The quantification of GFP fluorescence revealed that presence of the CKⅡ inhibitor prevented the directed nuclear enrichment of the NLS*P*-GFP (Figure 2D). Comparable results were obtained for cells expression the NLS*P*-GFP(T100A) mutant. An equal distribution comparable to GFP was observed (data not shown).

To study the relevance of the identified bipartite NLS for the subcellular distribution of the HBV polymerase cells were transfected with an expression construct encoding for HBV polymerase and analyzed by confocal immunofluorescence microscopy or subjected to cell fractionation. The immunofluorescence microscopy shows that in HBV P overproducing cells a fraction was found within the nucleus. However, in cells overexpressing the T100A mutant that destroys the CKⅡ phosphorylation site the nuclear-localized fraction disappeared and the P was exclusively found in the cytoplasm (Figure 2E). Western blotting analysis of the cytoplasmic and of the nuclear fraction confirmed that in addition to the cytosolic fraction a significant amount of P was detectable in the nucleus. However in cells treated with DMAT or overexpressing the T100A mutant of the putative CKⅡ phosphorylation site no P-specific signal was detectable in the nuclear fraction (Figure 2F).

Taken together these results indicate that the HBV polymerase harbors a bipartite nuclear localization signal which functionality is dependent on CKⅡ-mediated phosphorylation.

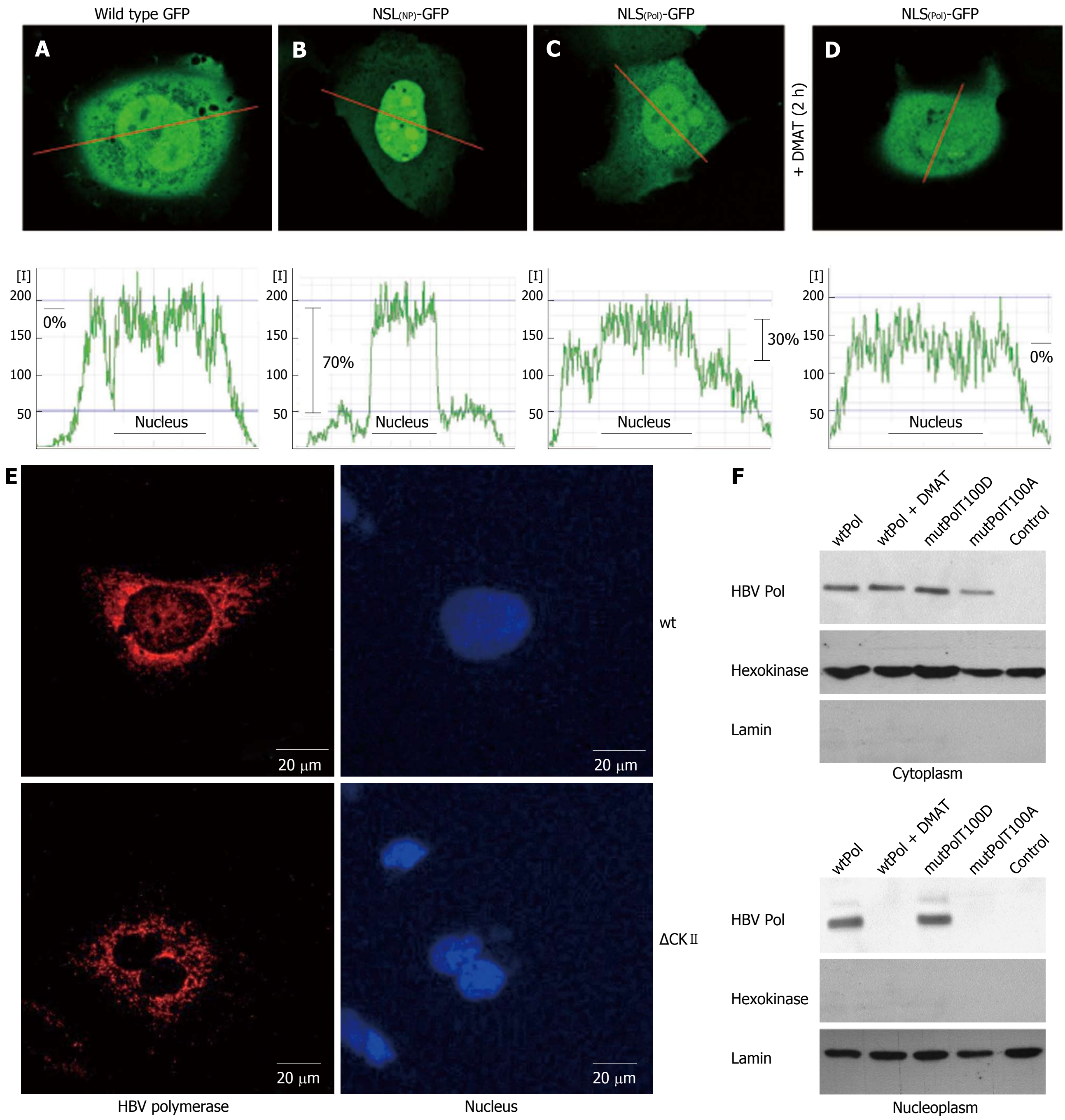

Figure 2 Hepatitis B virus polymerase harbors a functional nuclear localization signal in the terminal protein domain. A-D: HuH-7 cells were transfected with (A) the negative control wild type green fluorescent protein (GFP) (pEGFP-N1), (B) a positive control: GFP fused to a prototype bipartite nuclear localization signal (NLS) of human nucleoplasmin, (C) GFP fused to the putative bipartite NLS of hepatitis B virus (HBV) P protein, (D) the same as (C) but cells were treated with 10 × IC_{50} of casein kinase Ⅱ (CKⅡ) inhibitor 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) 2 h prior analysis. The fluorescence was measured in living cells by confocal laser scan analysis. The central layer (out of 6) was quantitated along the red indicated line and displayed in the corresponding graph of the lower panel as relative fluorescence intensity [I]. Differences of mean fluorescence intensities in the cytoplasm and within the nucleus (indicated as black line in the graph) were calculated and are indicated in percent. One representative cell for each fusion protein is shown; E: Confocal immunofluorescence microscopy of HuH-7 cells transfected with an expression vector encoding wt P or the mutant ΔCKⅡ (= T100I) that is not phosphorylated by CKⅡ. For detection of P (red) a rabbit-derived spacer domain-specific serum was used. Nuclei were stained with DAPI (blue); F: HuH-7 cells were transfect with the indicated expression vectors and left untreated or treated with 10× IC_{50} of CKⅡ inhibitor DMAT 5 h prior analysis. Transfection with pCDNA.3 served as control. Cells were lysed, the cytosolic and nuclear fraction were isolated by differential centrifugation and analyzed by western blotting. For detection of P a TP-domain specific serum was used. Detection of hexokinase and of lamin served as loading control and as control for the purity of the subcellular fractions. All experiments were performed in triplicate. One representative is shown.

Binding of karyopherin-α2 to TP depends on CKⅡ mediated phosphorylation

Karyopherin-α2 is an essential factor for NLS-mediated nuclear import. Therefore, it was investigated whether the data described above are reflected by an increased binding of karyopherin-α2 to *in vitro* phosphorylated TP as compared to unphosphorylated TP. Equal amounts of terminal protein domain were immobilized on two columns.

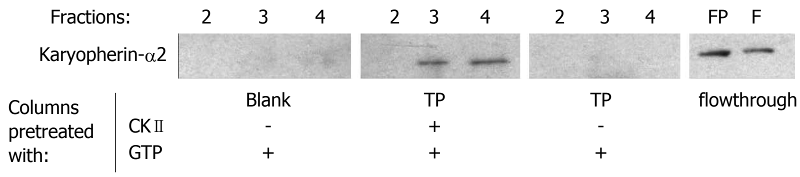

Figure 3 Binding of karyopherin-α2 to terminal protein depends on casein kinase Ⅱ mediated phosphorylation. The interaction of soluble protein fraction of HuH-7 cells to immobilized terminal protein (TP) domain was investigated by western blotting of the sodium chloride eluted fractions 2-4. Karyopherin-α2 bound only to the casein kinase Ⅱ (CKⅡ) pre-treated TP column (upper panel). FP: Flow through of the CKⅡ treated TP column; F: Flow trough of the untreated TP column.

One column was *in vitro* phosphorylated by CKⅡ and its substrate GTP the other and a blank control column were treated equally without the addition of the kinase. The pretreated columns were equilibrated and loaded with the desalted 75% (w/v) ammonium sulfate fraction of HuH-7 cell lysate. Finally, binding partners were eluted by sodium chloride and the eluted fractions were analyzed by Western blotting using a karyopherin-α2 specific antibody. Interestingly, karyopherin-α2 was only found in the eluate of the column with CKⅡ pre-treated TP (Figure 3). This indicates clearly that *in vitro* binding of karyopherin-α2, the key enzyme for nuclear import, is dependent on CKⅡ phosphorylation of the terminal protein.

Inhibition of CKⅡ impairs HBV replication in primary Tupaia hepatocytes

To study the relevance of the NLS and of the CKⅡ phosphorylation site for the HBV life cycle the HBV P protein was mutated based on a recombinant 1.2 fold HBV genome (subtype ayw) by site directed mutagenesis. The changes in the DNA sequence did not affect other reading frames or regulatory elements.

In the NLS-deficient mutant (ΔNLS) the NLS is inactivated by manipulating the basicity of the downstream cluster to K105D and K106S. The putative CKⅡ recognition site on the P protein is destroyed by a T100I substitution (ΔCKⅡ), whereas a pseudo-phosphorylated mutant was generated by a T100D conversion (CKⅡ*). However, the attempt to produce mutant virus by transfection of HepG2 or HuH-7 cells with the respective 1.2 fold genomes failed in case of ΔNLS and of the ΔCKⅡ mutant (Figure 4A). In both cases significant less virus was produced (about hundredfold) as compared to the wt genome or the mutant encoding the *CKⅡ** mutant. In a transfection experiment the nuclear import is not the limiting step. It can be concluded that mutations affecting the integrity of the NLS motive or of the CKⅡ phosphorylation site cause secondary effects affecting the functionality of the polymerase. Therefore a direct analysis of the relevance of the NLS and of the CKⅡ substrate domain based on mutated virus was not possible.

The *in vitro* data described above had shown that the functionality of the NLS identified in the TP-domain depends on the CKⅡ-dependent phosphorylation (Figure 2). To study the relevance of CKⅡ-dependent phosphorylation of the TP domain for HBV life cycle primary *Tupaia* hepatocytes were infected with wtHBV particles for 8 h. Adherent HBV particles were removed by trypsin treatment as described above. After infection the cells were grown for 36 h in the presence of the cell permeable small molecular CKⅡ inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4B). Moreover secreted viral particles were quantified by real time PCR. Both approaches revealed that inhibition of CKⅡ by DMAT caused a strong and significant reduction of virus replication. This was further confirmed by analysis of cccDNA formation in infected PTHs. Inhibition of CKⅡ by increasing concentrations of DMAT abolishes cccDNA formation (Figure 4B).

For a more detailed analysis primary Tupaia hepatocytes were infected as described above. One, two, four and seven days after infection the hepatocytes were grown for 36 h in the presence of the cell permeable small molecular CKⅡ inhibitor DMAT and the virus replication was analyzed by quantification of HBsAg and HBeAg secretion (Figure 4C and D). Both approaches revealed that inhibition of CKⅡ by DMAT at 1 and 2 d after infection caused a strong and significant reduction of virus replication, while inhibition after 4 and 7 d *pi* resulted only in a small reduction of virus replication.

To control the specificity of the observed effect the constitutively HBV expressing cell line HepG2.2.15 were instrumental. This cell line harbors a stably integrated HBV genome. Due to the stable integration of the genome the re-import of *de novo* synthesized genomes plays a minor role for maintaining the pool of transcriptional templates. Therefore, inhibition of polymerase import should exert a small effect. HepG2.2.15 cells were treated with DMAT, HBeAg and HBsAg secretion were analyzed by ELISA and virus secretion was quantified by LightCycler PCR (Figure 4B). Under these conditions inhibition of CKⅡ with DMAT did slightly but not significantly reduce virus secretion as compared to the solvent control (Figure 4B). Comparable results were obtained for the cell line HepAD38 (data not shown).

Taken together these data provide indirect evidence that the functionality of the NLS in the TP domain of P is required for the import of the viral genome into the nucleus.

DISCUSSION

It is well as established that HBV replicates its genome inside the nucleus (recent reviews[18,43,44]). This raises the question about the post entry transport of the genome complex to and about its final import into the nucleus.

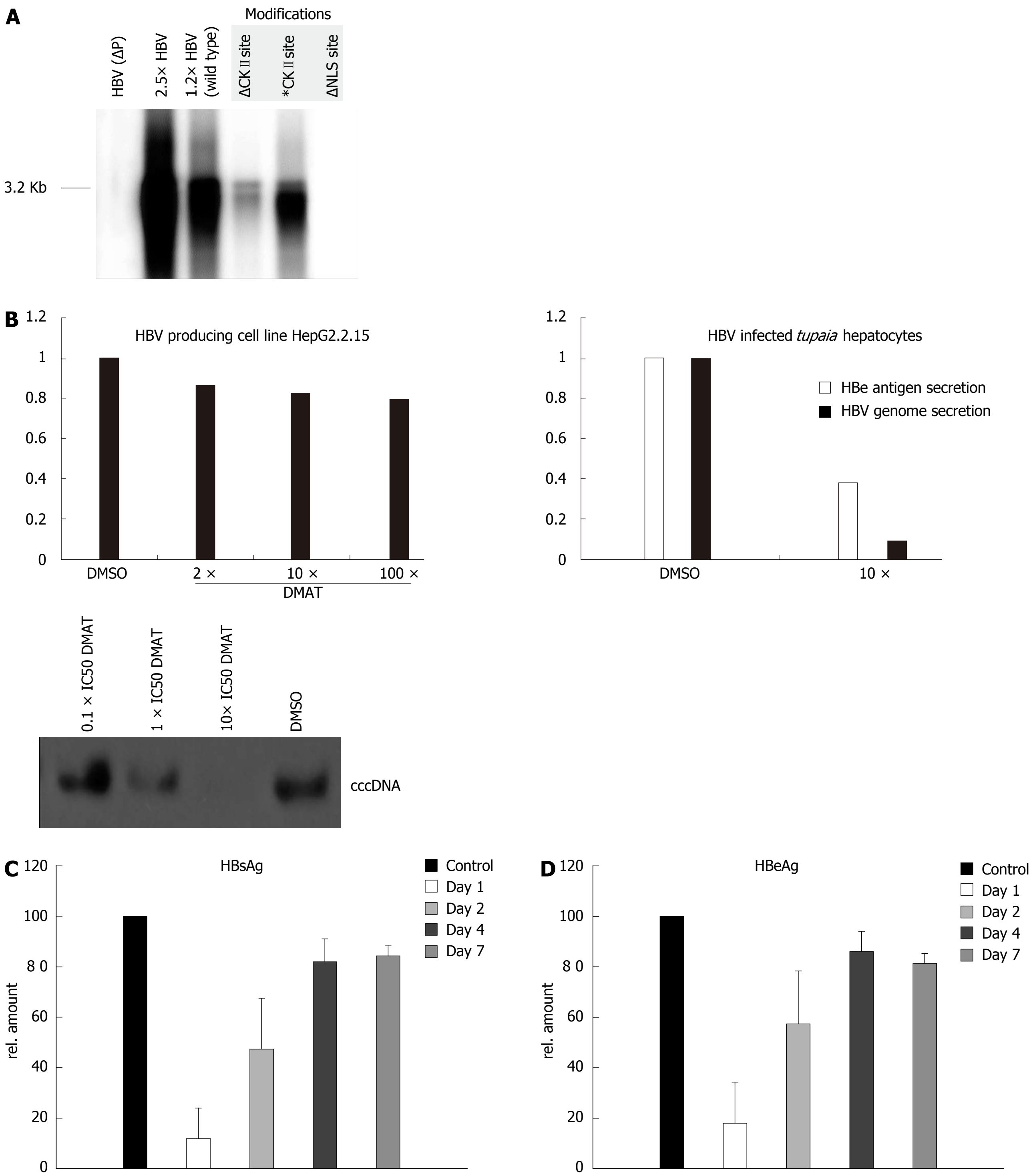

Figure 4 Casein kinase Ⅱ inhibition impairs hepatitis B virus replication in infected primary Tupaia hepatocytes. A: HuH-7 cells were transfected with mutant versions of a 1.2 fold hepatitis B virus (HBV) genome. After 5 d the secreted viral particles were precipitated with a HBs specific antibody and the containing 3.2 kb HBV genomes were visualized by the radioactive tracer [α-^{32}P] dCTP incorporated using the endogenous polymerase activity. The unphosphorylated form of the casein kinase Ⅱ (CKⅡ) recognition site in the P protein was simulated by a T100I substitution (Δ) and the pseudo-phosphorylation was simulated by a T100D substitution (*) in the 1.2 fold HBV wild type genome. The nuclear localization signal (NLS) was inactivated by mutating the downstream basic cluster (K105D and K106S) on the P protein. A 2.5 × HBV genome and a P deficient genome [HBV (P-)] served as controls; B: Primary *Tupaia* hepatocytes were infected with HepAD38 derived HBV and post infection treated for 36 h with solvent DMSO or 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) (10 × IC_{50}). Twelve days after infection the HBV genome secretion was measured by Lightcycler polymerase chain reaction. The cccDNA content of infected *Tupaia* hepatocytes that were incubated for 36 h with the indicated amounts of DMAT was visualized by Southern blot using a HBV specific probe. The specificity of DMAT incubation was analyzed by 2 h inhibitor pre-treatment of the stably HBV transfected cell line HepG2.2.15 followed by treatment of 36 h with 10 × IC_{50} CKⅡ inhibitor DMAT (IC_{50} in rat liver = 150 nmol/L) and genome secretion was compared to the solvent control DMSO measured by Lightcycler PCR. All experiments were performed in triplicate. The figure shows one representative experiment; C, D: Primary *Tupaia* hepatocytes were infected with HepAD38-derived HBV and at day 1, day 2, day 4 and day 7 treated for 36 h DMAT (10 × IC_{50}). Twelve days after infection the HBV replication was measured by HBeAg or HBsAg-specific enzyme linked immunosorbent assay. The bars represent the standard deviation.

Previous reports discussed whether the final import of the assembled nucleocapsid in the nucleus harboring the genome complex can occur[17]. More recent reports provide evidence that the mature nucleocapsid disassembles in the nuclear pore complex and the final import of the genome complex could be mediated by an association with HBcAg oligomers that harbor in their C-terminal domain a NLS[20,21,44-47]. For DHBV it was described that completition of plus-strand DNA synthesis triggers genomic DNA deproteinization and conformational changes of the nucleocapsid. This could lead to the exposure of a NLS within the core and thereby could enable the import of the rcDNA[48]. In this context it is interesting to mention that the presence of the identified NLS is not conserved for the genus avihepadnavirus.

Recently we developed cell permeable HBV nucleocapsids as a vehicle for gene transfer (Brandenburg *et al*[13]) Based on this system it was observed that neither HBcAg dimers nor nucleocapsids were visible in the nucleus of TLM-nucleocapsid treated cells although an efficient expression of the packaged, P-linked genome occurred suggesting that neither the nucleocapsid nor HBcAg dimers mediate the final import of the genome complex. Therefore the question arose about an alternative import mechanism: with about 90 kDa the covalent complex of HBV polymerase and genome clearly exceeds the size that freely can pass the nuclear pore complex.

In this context it is interesting that previous in vitro experiments have shown that the HBV genome complex can be efficiently imported into the nucleus. However if the complex is deproteinized, the naked genome fails to enter efficiently the nucleus[23]. These data suggest that the genome-linked polymerase could be relevant for the nuclear entry process.

The bipartite NLS identified in the TP domain of P could mediate the entry of the genome complex into the nucleus. The functional analysis of the genome complex revealed that the predicted NLS indeed has the potential to act as a nuclear localization signal. However, compared to other nuclear localization signals the TP-derived NLS is not a strong signal. This might reflect the different functions of P[18]. On the one hand P recognizes in the cytoplasm the de novo-synthesized 3.5 kb mRNA[43,49] and on the other hand the genome associated Pol is assumed to mediate by its NLS the entry of the genome complex into the nucleus. It is obvious that a too strong NLS signal might counteract the RNA-recognizing function in the cytoplasm. Yet, it was described that in duck HBV replicating cells in addition to the encapsidated polymerase non-encapsidated polymerase exists[24]. The major fraction of the non-encapsidated duck HBV polymerase is found in the cytoplasm a smaller fraction however can be detected within the nucleus[24]. Interestingly, in cells overexpressing HBV polymerase in the absence of other viral proteins a fraction of HBV polymerase is found within the nucleus, co-localized with the p11 protein of PML bodies[22].

A further interesting feature is the CKⅡ phosphorylation site localized in the center of the bipartite NLS. The functionality of the HBV polymerase-derived NLS depends on the CKⅡ-mediated phosphorylation. CKⅡ is not a very tightly regulated kinase[50]. It can be assumed that CKⅡ exerts a housekeeping phosphorylation function[51]. If subcellular localization and function of HBV polymerase is subjected to a tight control, it is not likely that CKⅡ exerts this function. It is tempting to speculate that phosphatases could play an important role to regulate the subcellular localization and thereby function of HBV polymerase. CKⅡ phosphorylation is reported to influence subcellular localization of various nuclear proteins[52]. For example CKⅡ phosphorylation upstream of the NLS of simian virus 40 T-antigen enhances its nuclear import up to 40 fold[53]. But immediate phosphorylation one or two amino acid upstream of the crucial amino acid of classical monopartite NLS seems to have inhibitory effects on karyopherin binding due to a disturbance of the NLS basicity[54]. In case of bipartite nuclear localization signals this correlation is not evident. For example the spacer of the functional bipartite NLS of the *Agrobacterium tumefaciens* protein nopaline contains four negative charged aspartates, one even immediate located ate the downstream basic cluster[55]. On the other hand an increase of the hydrophobicity of the 10-12 amino acid spacer seems to decrease its functionality[56].

In transfection experiments it was found that destruction of the NLS or of the CKⅡ-site almost completely abolishes HBV replication. Under these experimental conditions the import of the viral genome into the nucleus does not represent the limiting step. The plasmid DNA freely moves into the nucleus. However this observation suggests that perturbation of this domain has further effects on the polymerase function. Since the ΔNLS and ΔCKⅡ-mutants were replication deficient it was not possible to study the replication of the respective mutant viruses. The in vitro data however have shown that the functionality of the TP-derived NLS requires the functionality of CKⅡ. Based on this it could be shown that inhibition of CKⅡ in the early phase of the infection abolished the establishment of HBV infection while inhibition of CKⅡ in a later phase of the infection or in a stable system had no effect on HBV replication.

In conclusion we demonstrate the presence of a bipartite NLS within the TP domain of P and provide evidence for a novel model describing the import of the HBV genome into the nucleus.

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COMMENTS

Background

Human hepatitis B virus (HBV) enters the cell by receptor mediated endocytosis and at the end of this process the nucleocapsid that harbours the viral genome is released into the cytoplasm and transported towards the nuclear pore complex. Establishment of a productive viral infection requires the transport of the HBV genome that is covalently linked to the polymerase into the nucleus. In the

nucleus the partial- double stranded DNA genome is converted to covalently closed circular (ccc) double stranded DNA.

Research frontiers

The phase of nuclear entry is not fully understood. It is discussed that the intact viral capsid shuttles the genome-polymerase complex into the nuclear basket of the nuclear pore complex. Here, after a partial disassembly of the nucleocapsid the polymerase-linked genome is released. The polymerase-genome complex however is too big to pass freely through the nuclear pore complex. This raises the question about the import mechanism.

Innovations and breakthroughs

The identification and characterization of a bipartite NLS in the HBV polymerase that harbours a phosphorylation site for casein kinase Ⅱ (CKⅡ) is described in this manuscript. The integrity of the phosphorylation site is crucial for the functionality of the NLS. Moreover, inhibition of CKⅡ prevents karyopherin α2 from binding to the polymerase. Thereby the import of the polymerase is impaired resulting in inhibited cccDNA formation that prevents the establishment of the viral infection. The data identify novel structural and functional prerequisites for the establishment of HBV infection.

Applications

The data describe a potential novel target for antiviral that could block the establishment of a HBV-infection.

Peer review

In this work, they identified a novel NLS located in the terminal protein domain of HBV polymerase and defined a CKⅡ phosphorylation site (threonine) which is adjacent to the NLS. This paper is well written.

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ORIGINAL ARTICLE

Addicts with chronic hepatitis C: Difficult to reach, manage or treat?

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Abstract

AIM: To assess the acceptance, safety and efficacy of care and treatment for chronic hepatitis C (CHC) in drug addicts.

METHODS: We designed a multidisciplinary, phase IV prospective cohort study. All illicit drug users (IDUs) visited a Territorial Addiction Service (SerT) in the District of Brescia, and hepatitis C antibody (HCVAb) testing positive were offered as part of a standardised hepatologic visit in our Gastroenterology Unit. Patients with confirmed CHC and without medical contraindications were administered peginterferon alfa-2b 1.5 μg/kg per week plus ribavirin (800-1400 mg/d) for 16-48 wk. All IDUs were unselected because of ongoing addiction and read and signed an informed consent form. Virologic responses at weeks 4 and 12 of therapy, at the end of treatment and 24 wk after the end of treatment were the main measures of efficacy. Adherence was estimated according to the 80/80/80 criteria.

RESULTS: From November 2007 to December 2009, 162 HCVAb+ IDUs were identified. Sixty-seven patients (41% of the initial cohort) completed the diagnostic procedure, and CHC was diagnosed in 54 (33% of the total). Forty-nine patients were offered therapy, and 39 agreed (80% of acceptance rate). The prevalent HCV genotype was type 1, and the HCV RNA baseline level was over 5.6 log/mL in 61% of cases. Five patients dropped out, two because of severe adverse events (SAEs) and three without medical need. Twenty-three and 14 patients achieved end of treatment responses (ETRs; 59%) and sustained virologic responses (SVRs; 36%), respectively. Thirty-one patients were fully compliant with the study protocol (80% adherence). The prevalence of host and viral characteristics negatively affecting the treatment response was high: age over 40 years (54%), male gender (85%), overweight body type (36%), previous unsuccessful antiviral therapy (21%), HCV genotype and viral load (60% and 62%, respectively), earlier contact with HBV (40%) and steatosis and fibrosis (44% and 17%, respectively). In a univariate analysis, alcohol intake was associated with a non-response (P = 0.0018, 95%CI: 0.0058-0.4565).

CONCLUSION: Drug addicts with CHC can be successfully treated in a multidisciplinary setting using standard antiviral combination therapy, despite several "difficult to reach, manage and treat" characteristics.

Key words: Chronic hepatitis C; Addiction; Antiviral therapy; Interferon; Multidisciplinary

Core tip: The paper reports results from a clinical trial on the management of chronic hepatitis C (CHC) in illicit drug users (IDUs). Two key elements characterise the trial: (1) the study was performed by a multidisciplinary team; and (2) the patients were unselected because of ongoing addiction. We assessed the acceptance of care and treatment for CHC among IDUs, who are classically considered to be a "difficult to reach and manage" group. For the IDUs accepting antiviral treatment, we analysed results on safety, efficacy and adherence and on the prevalence of negative prognostic factors affecting the virologic response to address whether IDUs are also "difficult to treat" patients.

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INTRODUCTION

Hepatitis C virus (HCV) is estimated to chronically infect more than 180 million people worldwide, with approximately 4 million carriers in Europe alone[1]. The prognosis of chronic hepatitis C (CHC) is related to fibrosis progression, and the development of cirrhosis varies from 5%-25% over an average period of 30 years[2]. According to a recently validated mathematical model, morbidity and mortality from HCV are expected to rise in 2010 and to peak between 2030 and 2035[3]. The main reasons for this negative forecast are the low rates of screening for HCV and of treatment for CHC. The World Health Organization has defined chronic infection with HCV as a public health problem of primary importance, and during a consensus meeting in May 2010, all health authorities were invited to strive to prevent, identify and rapidly treat the infection[1].

In developed countries, HCV is mainly transmitted by needles during drug injections, and illicit drug users (IDUs) are considered to be the largest group affected by HCV, accounting for 20%-50% of cases of chronic infection[4]. A recent paper estimating viral transmission showed, for the first time, that HCV "super-spreading" is led by IDUs. According to this estimation model, each infected IDU is likely to infect approximately 20 other people, half of whom will be infected within 2 years of the initial infection[5].

International authorities on liver diseases (*i.e.*, the National Institutes of Health since 2002, the American Gastroenterological Association since 2006 and the American Association for the Study of the Liver since 2009) recommend the treatment of CHC in IDUs and encourage clinical studies in chronically infected IDUs "to evaluate the safest and most effective treatment, factors favouring compliance, risk of relapse, side-effect profile and the impact of methadone maintenance treatment"[6-8].

Despite international recommendations, several barriers to treating IDUs persist not only among physicians but also among IDUs[9,10]. Physicians' concerns mainly include IDUs' chaotic lifestyle; IDUs' possibly poor adherence to treatment; difficulties in the management of the psychiatric side effects of treatment, which are believed to be more frequent among IDUs; and the risk of re-infection after HCV eradication[10]. The risk of relapse into addiction due to interferon (IFN)-driven mood changes and the use of needles in CHC therapy, is also described as a relative contraindication to IFN treatment in IDUs, although the data from prospective trials on this risk are scarce[11,12]. Concerns about antiviral therapy for CHC are also present among IDUs: their conception of illness and death is often different, for cultural reasons, from the beliefs of the general population, and information on the natural history and treatment challenges of HCV is inexact or incomplete[10]. Moreover, factors including precarious working conditions, a lack of fixed adobe, undocumented migrant status and social isolation can affect access to care and treatment for liver diseases, and special efforts may be necessary to reach IDUs in their social environment[13]. In our recent review on the subject[14], we report that due to the barriers to treatment, most published prospective trials on the treatment of CHC in IDUs involve limited numbers of patients, ranging from 11-71, have no standardised intervention protocol and are mainly restricted to abstinent patients.

As an overview, IDUs are perceived as patients who are difficult to reach for social reasons and difficult to manage because of lifestyle. Moreover, no previous study has assessed whether IDUs are also difficult to treat because of the presence of negative prognostic factors, either viral or host-related, affecting the rate of success of antiviral therapy. For these difficulties to be addressed, and to successfully be able to contact, manage and treat these patients, a multidisciplinary approach is mandatory. This approach should involve health professionals engaged in the management of addiction and dedicated hepatologists with a highly personalised approach to patient care.

We performed a prospective clinical study designed to maximise IDUs' access to treatment for HCV infection by involving both the physicians directly engaged in the management of addiction and the specialised hepatologist in our unit and by avoiding "*a priori*" exclusion criteria for antiviral therapy of active IDUs. The main objectives of the study were to specifically evaluate the rate of access to clinical care; the acceptance, safety and efficacy of antiviral treatment for CHC; and the prognostic factors for responses to standard antiviral therapy in a large cohort of IDUs, who were unselected because of ongoing addiction.

MATERIALS AND METHODS

Study design

We designed a multidisciplinary, phase IV prospective

cohort study. The multidisciplinary approach was ensured by close collaboration between six Territorial Addiction Services (SerT) of the Local Health Authority of the District of Brescia (ASL) and our Gastroenterology Unit (GU, Spedali Civili and University of Brescia). The physicians of the SerT were responsible for the identification of patients with hepatitis C antibody (HCVAb) positivity among those individuals visiting the SerT clinic. Based on the protocol definition, patients with "ongoing addiction problems", actively using illicit drugs and/or alcohol or in a supportive/substitution treatment program, were all considered to be subjects. The SerT physicians were also responsible for collecting all demographic, social, psychological and addiction data in a standardised case report form. The patients selected by the SerT physicians were instructed to call a dedicated telephone number to make an appointment for an initial standard hepatologic evaluation in the GU, including a medical visit, laboratory tests and ultrasound evaluation. The aims of the hepatologic evaluation were to confirm HCV-related chronic hepatitis, to assess the severity of liver disease and to evaluate eligibility for antiviral treatment. A liver biopsy was not routinely performed. The patients with confirmed CHC and meeting standard criteria for HCV therapy[8] were offered antiviral treatment with pegylated interferon and ribavirin, according to the study protocol for treatment, and were asked to sign an informed consent form. To improve adherence, a mobile telephone number was activated for all patients on antiviral treatment, with a physician on call every day from 8 a.m. to 1 p.m.

Selection criteria

The inclusion criteria were as follows: over 18 years of age, HCV RNA detected with a sensitive polymerase chain reaction (PCR) (cut-off of determination 50 IU/mL; COBAS Amplicor HCV test, Roche Diagnostics, Branchberg, NJ, United States) and confirmed on at least two occasions over a period of 6 mo, compensated liver disease (Child-Pugh score $\leqslant$ 5), absence of major medical contraindications to antiviral therapy (including malignancies, severe cardiac illness and uncontrolled psychiatric condition), willingness to avoid pregnancy during the entire treatment period and during the 6 mo after the last ribavirin dose intake, ability to read and sign a written informed consent form and willingness to adhere to the study protocol for treatment. Patients with suspected or confirmed idiosyncratic reactions to interferon or ribavirin were excluded.

Treatment protocol

The study protocol for treatment consisted of peginterferon alfa-2b (12 kDa) 1.5 μg/kg per week plus ribavirin 800-1400 mg according to body weight (800 mg for < 65 kg, 1000 mg for 65-80 kg, 1200 mg for 81-105 and 1400 mg for > 105 kg) divided into two daily administrations.

The duration of the treatment was 24 wk for HCV genotypes 2/3 and 48 wk for HCV genotypes 1/4. The achievement of a rapid virologic response (RVR; HCV RNA < 50 IU/mL at week 4 of therapy) was regarded as an indication for short-term therapy in all naïve patients fulfilling the criteria of no dose reduction during the first 4 wk of therapy, low baseline viral load (HCV RNA < 600000 IU/mL) and the absence of cirrhosis. The short-term scheme consisted of 16 wk for HCV genotypes 2/3 and 24 wk for HCV genotypes 1/4. Treatment was discontinued prematurely, according to international rules[8] (at week 12 if the HCV RNA level drops by < 2 Log and at week 24 if the HCV RNA level is > 50 IU/mL); in the case of virologic breakthrough; in the presence of severe adverse events (SAEs); or upon patients' request, with no need for explanation. In the case of a virologic breakthrough, HCV genotyping [line probe assay (LIPA), Bayer HealthCare, Tarrytown, NY, United States] was performed to exclude mixed/new HCV infections.

Adverse reactions to interferon and/or ribavirin were managed according to international guidelines[8], and the use of erythropoietin and leucocyte growth factors was allowed, according to the current Italian Drug Agency (AIFA) recommendations.

The timetable of the study was as follows: a medical visit including a general physical examination, an assessment of body mass index, the administration of AUDIT-C for screening for at-risk alcohol-related behaviour and a Hamilton Test for scoring anxiety and depression. The measurement of the complete blood count and the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) levels was requested every 4 wk during treatment and 24 wk after the end of therapy. Blood tests, including for thyroid function (FT4 and TSH), autoimmunity (ANA, AMA, LKM and ASMA) and liver function (albumin, PT and bilirubin), were mandatory at the beginning and every 12 wk of treatment. Quantitative and qualitative assays for HCV RNA were requested at week 0-4-12 and at week 4-12-24 of treatment, at the end of therapy and 24 wk after the end of therapy, respectively.

Outcome measures

Access to liver care was calculated as the proportion of IDUs attending the first hepatologic visit and completing the diagnostic procedure among all HCVAb+ IDUs screened by the SerT and showing interest in this opportunity. Access to therapy was evaluated as the proportion of patients starting antiviral therapy among the eligible patients. The main measure of safety was the rate of withdrawal for SAEs, according to the Common Terminology Criteria for Adverse Events v3.0[15]. The main outcome measure of efficacy was the sustained virologic response (SVR; HCV RNA persistently < 50 IU/mL 24 wk after treatment discontinuation) among the treated patients. Secondary efficacy measures were the achievement of an RVR, an early virologic response (EVR; HCV RNA < 50 IU/mL at week 12 of therapy) and an end of treatment response (ETR; HCV RNA < 50 IU/mL at the end of therapy). Adherence was estimated according to the 80/80/80 criteria (80% of pegylated interferon,

Table 1 Main baseline characteristics of 162 hepatitis C antibody+ illicit drug users selected by physicians operating in six Territorial Addiction Service in the District of Brescia and comparison with 39 hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

Patient characteristics	Selected by SerT (*n* = 162)	Accepting therapy (*n* = 39)	*P* value
Male gender	135 (83)	27 (69)	0.8152
Age, yr, mean ± SD	38 ± 7	39 ± 6	0.8888
Spoken language: Italian	152 (94)	33 (85)	0.0912
Place of birth			0.1502
Italy	147 (91)	32 (82)	
EU	5 (3)	2 (5)	
Non-EU	10 (6)	5 (13)	
Level of education	(*n* = 151)		0.8320
≤ 8 yr of school	118 (79)	30 (77)	
High school diploma	32 (21)	8 (21)	
University degree	1 (0)	1 (2)	
	(*n* = 149)		1.0000
Unemployed	49 (33)	13 (33)	
Chronic associated conditions	41 (25)	11 (28)	0.6888
Cardiovascular	5 (3)	2 (5)	
Respiratory	4 (2)	1 (3)	
Allergic	2 (1)	1 (3)	
Psychiatric	19 (12)	3 (8)	

SerT: Territorial Addiction Service.

Table 2 Type of addiction and opiate substitution treatment among the hepatitis C antibody+ illicit drug users selected by the Territorial Addiction Service and comparison with hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

	Selected by SerT (*n* = 162)	Accepting therapy (*n* = 39)	*P* value
Alcohol			
Active	11 (7)	4 (10)	0.4972
Partial remission	5 (3)	0 (0)	0.5867
Total remission	23 (14)	10 (26)	0.0944
Cannabis			
Active	7 (11)	2 (5)	0.0590
Partial remission	3 (2)	1 (3)	0.5811
Total remission	4 (2)	3 (8)	0.1344
Cocaine			
Active	33 (20)	3 (8)	0.0038
Partial remission	6 (4)	1 (3)	1.0000
Total remission	39 (24)	12 (31)	0.4150
Heroin			
Active	48 (30)	6 (15)	0.1059
Partial remission	19 (12)	5 (13)	0.7885
Total remission	74 (46)	23 (59)	0.0647
Duration of intravenous drug use, yr, mean ± SD (range)	(*n* = 98) 13 ± 8 (7-34)	(*n* = 33) 13 ± 9 (6-32)	0.8588
Opiate substitution treatment	126 (78)	28 (72)	0.4089
Methadone, mg, mean ± SD	107 (66), 41 ± 22	19 (60), 46 ± 26	0.0642
Buprenorphine, mg, mean ± SD	19 (12), 5 ± 3	9 (23), 6 ± 4	0.0751

A smoking habit was concomitant in 100% of patients: < 5 cigarettes/d in 4%, 5-10 cigarettes/d in 12%, 11-20 cigarettes/d in 54% and > 20 cigarettes/d in 30%. SerT: Territorial Addiction Service.

80% ribavirin cumulative dosage and 80% of the duration of therapy[16]).

Ethics

The entire study design was evaluated by the Ethics Committee of Spedali Civili of Brescia and fully approved on July 31st, 2007. The study was registered with EudraCT, number 2008-001283-37.

Statistical analysis

Treatment efficacy was measured according to the intention to treat (ITT) criteria, including all patients who had received at least one dose of interferon and one dose of ribavirin after signing the informed consent form. For statistical analyses, an unpaired t-test and Fisher's exact test were used when appropriate using GraphPad Prism, version 5.0 (Graph Pad Software, Inc., San Diego, CA, United States). Logistic regression analysis was performed to assess the effect of baseline features on efficacy and was completed using Stata software (version 7, StataCorp LP, College Station, TX, United States). A *P* value < 0.05 was accepted to reject the null hypothesis.

RESULTS

Identification of the cohort

From November 2007 to December 2009, a cohort of 162 HCVAb+ IDUs was identified by six SerT in the District of Brescia. Most patients were Italian males with a low level of education. At the time of recruitment, one third of the patients were unemployed, and one quarter had comorbidities (Table 1). The prevalent type of addiction was intravenous injection of heroin, with a mean duration of 13 years. Most patients were on opiate substitution treatment, with 66% on methadone and 12% on buprenorphine. All patients in methadone maintenance therapy received a dose lower than 100 mg/d (Table 2). Most IDUs who received information about HCV infection from health operators, the press or television were not confident about their knowledge and had moderate worries about the side effects of HCV therapy (Table 3).

Access to care and treatment

Patient disposition, according to the study protocol, is reported in Figure 1. Access to the first hepatologic work-up was observed in 106 patients, which was 65% of the initial cohort. Although 56 IDUs expressed interest in the opportunity for a dedicated medical examination to a SerT doctor, these individuals never called our clinic for an appointment. Sixty-seven patients completed the diagnostic procedure, or 41% of the initial cohort, corresponding to 63% of patients visiting our clinic for an initial evaluation. Patients who did not adhere to the diagnostic protocol (39 IDUs) were all contacted by telephone by a physician (BZ), and these patients all preferred to postpone the medical procedures because of

Table 3 Attitudes toward/knowledge about hepatitis C virus infection among the hepatitis C antibody+ illicit drug users selected by the Territorial Addiction Service in comparison with hepatitis C antibody+ illicit drug users accepting antiviral therapy *n* (%)

Patient attitudes/knowledge	Selected by SerT (*n* = 162)	Accepting therapy (*n* = 39)	*P* value
Source of HCV information	(*n* = 150)	(*n* = 33)	NS
Other HCV patients	44 (29)	11 (33)	
Health operators	72 (48)	18 (55)	
Press	54 (36)	14 (42)	
Web	15 (10)	6 (18)	
Television	62 (41)	16 (48)	
None	25 (17)	6 (18)	
Feelings toward information			
Complete	(*n* = 139) 72 (52)	(*n* = 32) 15 (47)	0.6964
Confident	(*n* = 131) 30 (23)	(*n* = 31) 16 (52)	0.0033
Reassuring	(*n* = 130) 68 (52)	(*n* = 28) 14 (50)	0.8381
Attitudes toward HCV therapy			
Total fright	(*n* = 129) 3 (2)	(*n* = 29) 0 (0)	1.0000
Moderate worries	(*n* = 141) 102 (78)	(*n* = 32) 25 (78)	0.5271
Positive expectations	(*n* = 125) 70 (56)	(*n* = 28) 18 (64)	0.5271

HCV: Hepatitis C virus; SerT: Territorial Addiction Service.

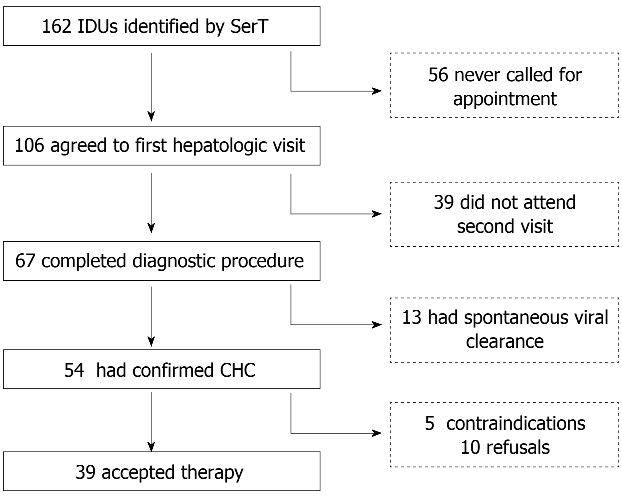

Figure 1 Patient disposition according to the study protocol. CHC: Chronic hepatitis C; IDU: Illicit drug user; SerT: Territorial Addiction Service.

other priorities.

CHC was confirmed in 54 IDUs (33% of the total), of which 13 patients had a confirmed HCV RNA-negative test, with an estimated rate of spontaneous clearance of 19%. Five patients had medical contraindications to specific antiviral therapy (two cases of decompensated cirrhosis, one case of hepatocellular carcinoma, one case of pregnancy and one case of uncontrolled severe psychiatric illness). The remaining 49 patients were offered specific treatment for CHC, and 39 accepted and signed the informed consent form (80% acceptance rate). Two patients never started treatment after signing the informed consent form and were thus excluded from the ITT analysis, and two patients, one with HCV genotype 1a and the other with HCV genotype 3a, relapsed after the end of therapy and asked for a second cycle of antiviral therapy (after a 6-mo wash-out period). We therefore report results for 39 treatments in 37 patients.

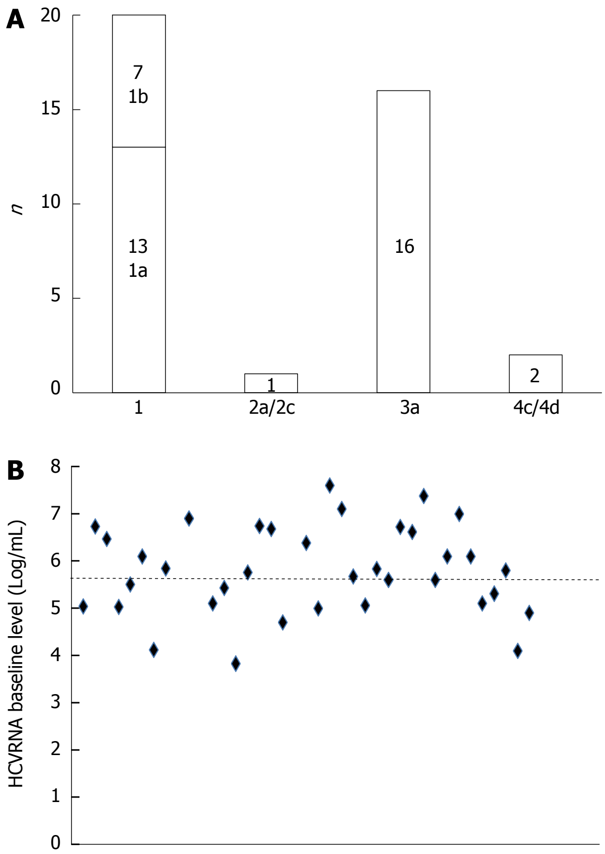

Figure 2 Hepatitis C virus genotypes (A) and baseline hepatitis C virus RNA levels (B) in 36 patients (the dotted line indicates the 5.6 Log/mL cut-off value for high viral load).

Baseline characteristics of treated patients

The virologic features of the treated patients are reported in Figure 2. The most represented HCV genotype was type 1 (13 patients with 1a and seven with 1b). The HCV RNA baseline level, available in 36 of 39 patients, was over 5.6 Log/mL in 22 cases (61%). In total, 36% of our patients were active illicit drug users, mainly using heroin; approximately one third had a history of depression; one quarter had a pathologic Hamilton score for anxiety or depression; four patients were addicted to alcohol; and seven patients had an AUDIT-C at-risk score (Table 4). As reported in Table 5, several prognostic factors negatively affecting the outcome of antiviral therapy for CHC were well represented among our treated IDUs. These factors included age over 40 years, male gender, overweight body type, previous unsuccessful HCV antiviral treatment, unfavourable HCV virologic genotype or viral load, earlier contact with HBV, steatosis and progression to cirrhosis.

Safety

Two SAEs occurred during the study period, leading to therapy discontinuation: a case of psychosis and a case of pneumonia with suspected tuberculosis at weeks 4 and 10, respectively. Three patients dropped out without

Table 4 Main baseline clinical and laboratory characteristics of treated illicit drug users

Characteristics	*n* = 39
BMI (kg/m^2), M (range)	24.3 (17.6-34.6)
Duration of HCV infection (yr), M (range)	5 (1-21)
Duration under 1 yr	14 (36)
Duration of IDU status (yr), M (range)	12 (1-32)
Active IDU	14 (36)
History of depression	11 (28)
Pathologic Hamilton score	
Anxiety	10 (26)
Depression	8 (21)
AUDIT-C at-risk score	7 (18)
Leucocytes (n/mm^3), M (range)	6960 (3960-11960)
Haemoglobin (g/dL), M (range)	15.5 (11.8-17.7)
Platelets	224 (106-421)
ALT index (value/u.l.n.), M (range)	2.5 (0.5-16.4)
AST index (value/u.l.n.), M (range)	2.0 (06-6.6)
GGT index (value/u.l.n.), M (range)	1.2 (0.3-13.9)

HCV: Hepatitis C virus; IDU: Illicit drug user; M: Male; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyltransferase.

Table 5 Main baseline features potentially affecting the response to antiviral therapy in treated illicit drug users

Features	Prevalence
Age over 40 yr	54%
Males	85%
BMI over 25 kg/m^2	36%
Previous unsuccessful interferon treatment	21%
Unfavourable HCV genotype (1 or 4)	60%
HCV viral load > 5.6 Log (IU/mL)	62%
HBcAb positivity	40%
Ultrasonography suggestive of steatosis	44%
Ultrasonography suggestive of cirrhosis	17%

HBcAb: Hepatitis B core antibody; HCV: Hepatitis C virus; BMI: Body mass index.

medical need and were lost to follow-up; all dropouts occurred within the first 8 wk of antiviral treatment. Sixteen (41%) and 17 (44%) patients needed a dose adjustment of pegylated interferon and ribavirin, respectively. In six patients (15%), the use of erythropoietin was offered. The use of leucocyte grown factors was not necessary for any patient. One patient became pregnant during the 6 mo after the end of therapy and decided on an abortion for personal reasons.

Efficacy

In the ITT analysis, 23 patients achieved an ETR (59%), and nine (23%) relapsed during the 6 mo after the end of therapy. Fourteen patients achieved an SVR (36%), seven of whom were infected with an unfavourable HCV genotype (Figure 3). The HCV RNA serologic clearance rates at weeks 4 and 12 are reported in Figure 4. Short-term therapy was offered to nine patients, according to the study protocol, and did not negatively affect the SVR rate based on univariate analysis.

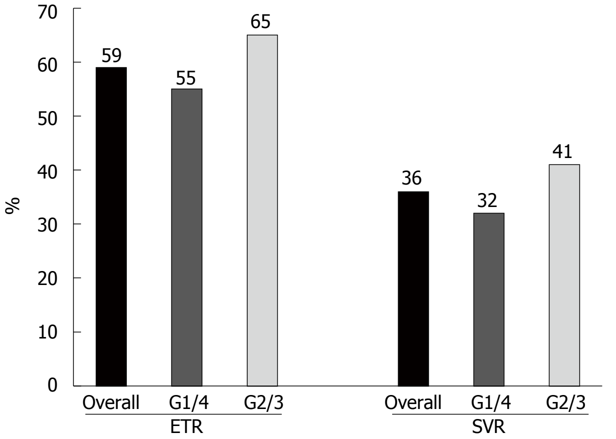

Figure 3 Percentage of end of treatment responses and sustained virologic responses in the entire cohort and according to Hepatitis C virus genotype (G). ETR: End of treatment response; SVR: Sustained virologic response.

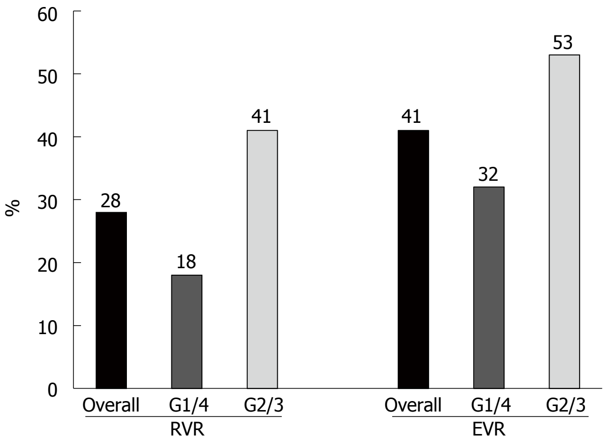

Figure 4 Percentage of rapid virologic responses and early virologic responses in the entire cohort and according to Hepatitis C virus genotype (G). RVR: Rapid virologic response; EVR: Early virologic response.

Adherence and prognostic factors for response

According to our adherence definition, 31 patients (80%) were compliant with the study protocol.

In the univariate logistic regression analysis, the active use of cocaine and/or heroin, ongoing substitution treatment, the type of substitution treatment, the presence of comorbidity, spoken language and male gender did not affect the rate of the SVR, whereas alcohol intake was associated with a non-response (P = 0.0018, 95%CI: 0.0058-0.4565), independent of the dose and type of alcoholic beverage.

DISCUSSION

Our study indicates that antiviral treatment for CHC in IDUs is safe and effective and that a multidisciplinary approach is a key element of the care of such patients. We have considered three main aspects of this issue to understand whether these patients are, as generally perceived, difficult to reach, manage or treat.

Are IDUS difficult to reach?

IDUs, together with migrants and prison inmates, are regarded as special population groups. As recommended by an official position paper on behalf of several Italian scientific societies[13], in such vulnerable people, specific intervention is mandatory to identify, prevent and treat chronic viral infections of the liver. In our approach, collaboration with the territorial services involved in the care of addicts was the key means of reaching IDUs (of whom 6%-15% were migrants) who were at risk of exclusion from medical care for social reasons. In total, 65% of IDUs with HCVAb positivity and identified by physicians of the SerT agreed to and received a dedicated medical visit that, even in the case of patients not receiving treatment, provided an instructive opportunity for counselling on HCV transmission, the prevention of liver complications, healthy lifestyle and available therapeutic protocols. In contrast, 56 IDUs (35% of the total cohort identified by the SerT) never called for an appointment, despite an initial statement of interest in the project. Moreover, after the first medical evaluation, 39 patients (37% of the patients who agreed to the first visit) never completed the diagnostic procedure, even after encouragement by direct telephone contact with a physician. Because of incomplete procedures, clinical and laboratory data were not sufficient to confirm an active HCV infection and to stage liver disease in 95 patients (59% of the initial cohort). Such a finding indicates that difficulties in reaching and motivating this population of patients persist even in the context of a well-organised multidisciplinary approach.

Are IDUs difficult to manage?

Concerns about treating IDUs are mainly due to suspicion of low adherence, the risk of SAEs (typically psychiatric) and the inability to follow therapeutic prescriptions[10]. In our study, adherence was high and comparable to the adherence reported for clinical trials in the general population[17,18]. The use of a psychiatric questionnaire to monitor depression and anxiety was well accepted; one patient received antidepressant therapy before starting antiviral treatment, and paroxetine was offered to another patient after 12 wk of antiviral treatment. The patient with a psychotic reaction completely recovered after the withdrawal of antiviral treatment without consequences or the need for psychiatric drugs. These data on the psychiatric safety of CHC treatment in IDUs, as previously suggested by other studies[19-22], are encouraging.

An important feature of our study was the inclusion of people who were actively addicted, with no period of mandatory abstinence; 36% of our enrolled patients continued to use illicit drugs (mainly heroin and cocaine) during the study protocol. Despite this "difficult to manage" characteristic, the data on safety, efficacy and adherence are encouraging. Moreover, logistic regression failed to demonstrate a negative correlation with the viral response to therapy in patients who were actively addicted during antiviral treatment. Only alcohol consumption was related to a lower SVR rate, and this finding confirms the role of alcohol consumption in the impairment of antiviral treatment efficacy, which has already been demonstrated in the general population[23].

Although IDUs are considered to be poorly motivated to undergo medical care, the multidisciplinary setting and strict collaboration among the different physicians involved in the care of the IDUs led to a high rate of access to therapy; 76% of patients with confirmed CHC started treatment. Such a rate was markedly higher than the rate previously reported in studies in the general population[10].

Are IDUs difficult to treat?

Adherence to treatment was high (80%), and despite few withdrawals for safety reasons, the overall SVR of 36% was lower than expected for the general population. This "efficacy" goal must be observed in light of several "difficult to treat" characteristics in our study population[24]. Among our IDUs, viral features such as HCV genotype 1 and a high baseline level of viremia were prevalent. Moreover, 40% of our patients tested positive for HBcAb[25]. Male gender, an overweight body type and an age over 40 were frequent. Other unfavourable factors affecting the virologic response were a relatively high prevalence of steatosis and cirrhosis in 44% and 17%, respectively, of patients. Most patients had been addicted for over 10 years, and no patient was identified and treated during the acute phase of the infection. In total, 21% of patients experienced the failure of at least one antiviral treatment for CHC. A few of these features are not modifiable (HCV genotype, viral load and gender), whereas other features could be modified by a more prompt strategy of intervention (younger age, shorter duration of infection and lower score of fibrosis).

In conclusion, IDUs with HCV-related CHC, actively using illicit drugs and/or opioid substitution treatment, can be successfully treated in a multidisciplinary setting with a standard antiviral combination of ribavirin and pegylated interferon, with good adherence and a good safety profile. IDUs' "difficult to reach, manage and treat" characteristics should not be used to contraindicate antiviral therapy. An appropriate multidisciplinary setting is a key factor in overcoming the "difficult" characteristics of these patients, with a strategic aim of reducing HCV circulation in the largest reservoir of this viral infection. Whether treatment will benefit from upcoming new antiviral agents is currently under study in our unit.

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COMMENTS

Background

Hepatitis C virus (HCV) infection is a common condition worldwide with prevalence of 3%. Illicit drug users (IDUs) are regarded as an important reservoir of this infection and as "super-spreaders". HCV infection is a progression disease possibly leading to chronic liver disease and ultimately to end stage liver disease. Is therefore important to identify strategy to eradicate infection particularly in the reservoir-population? Concerns about therapy of HCV infection in these populations are present in both physicians and IDUs.

Innovations and breakthroughs

The investigators report that within a multidisciplinary setting involving both liver and addiction specialists nearly half of identified HCV+ IDUs accept hepatologic counseling and nearly a quarter accept treatment. Eighty percent of treated patients are adherent to treatment according to 80/80/80 rule. Sustained virological response is achieved in a proportion similar of that reported in registration trials, is not influenced by ongoing addiction, but is negatively affected by alcohol consumption. Incidence of psychiatric and organic side effects is not different from that reported in the general population.

Applications

This article supports the concept that barriers to HCV therapy of IDUs can be overcome in the context of a multidisciplinary team, and that in this clinical context adherence and efficacy of therapy is similar as in the general population. The study highlights the point that the risk of HCV spreading by the super-spreaders IDUs can be reduced and that their habits can not be used as an argument to withhold antiviral therapy.

Peer review

The study includes challenging for ethical difficulty of HCV treatment. It's very interesting and authors may applaudable effort on this study. Of course, opposite opinions for HCV treatment on addicts may exist, nonetheless this study indicates possibility of HCV treatment for some addicts if patients can receive enough support from medical profession. Although this study may raise an ethical issue, it will give a strong impact to readers and make fascinating reading.

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ORIGINAL ARTICLE

Expression of hepatitis B virus 1.3-fold genome plasmid in an SV40 T-antigen-immortalized mouse hepatic cell line

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Abstract

AIM: To investigate the expression of the hepatitis B virus (HBV) 1.3-fold genome plasmid (pHBV1.3) in an immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression.

METHODS: Mouse hepatic cells were isolated from mouse liver tissue fragments from 3-5 d old Kunming mice by the direct collagenase digestion method and cultured *in vitro*. The pRSV-T plasmid was transfected into mouse hepatic cells to establish an SV40LT-immortalized mouse hepatic cell line. The SV40LT-immortalized mouse hepatic cells were identified and transfected with the pHBV1.3 plasmid. The levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant were determined by an electrochemiluminescence immunoassay at 24, 48, 72 and 96 h after transfection. The expressions of HBsAg and hepatitis B c antigen (HBcAg) in the cells were investigated by indirect immunofluorescence analysis. The presence of HBV DNA replication intermediates in the transfected cells and viral particles in the supernatant of the transfected cell cultures was monitored using the Southern hybridization assay and transmission electronic microscopy, respectively.

RESULTS: The pRSV-T plasmid was used to immortalize mouse hepatocytes and an SV40LT-immortalized mouse hepatic cell line was successfully established. SV40LT-immortalized mouse hepatic cells have the same morphology and growth characteristics as primary mouse hepatic cells can be subcultured and produce albumin and cytokeratin-18 *in vitro*. Immortalized mouse hepatic cells did not show the characteristics of tumor cells, as alpha-fetoprotein levels were comparable (0.58 ± 0.37 *vs* 0.61 ± 0.31, $P = 0.37$). SV40LT-immortalized mouse hepatic cells were then transfected with the pHBV1.3 plasmid, and it was found that the HBV genome replicated in SV40LT-immortalized mouse hepatic cells. The levels of HBsAg and HBeAg continuously increased in the supernatant after the transfection of pHBV1.3, and began to decrease 72 h after transfection. The expressions of HBsAg and HBcAg were observed in the pHBV1.3-transfected cells. HBV DNA replication intermediates were also observed at 72 h after transfection, including relaxed circular DNA, double-stranded DNA and single-stranded DNA. Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were detected in the supernatant.

CONCLUSION: SV40T expression can immortalize mouse hepatic cells, and the pHBV1.3-transfected SV40T-immortalized mouse hepatic cell line can be a new *in vitro* cell model.

Key words: SV40 T-antigen; Mouse hepatic cell; Hepatitis B virus 1.3-fold genome plasmids; Immortalized; Liposomes; Transfection

Core tip: This study established a new immortalized mouse hepatic cell line through the transfection of the pRSV-T plasmid. SV40 T-antigen (SV40LT)-immortalized mouse hepatic cells had the same morphology and biological characteristics as primary mouse hepatic cells. SV40LT-immortalized mouse hepatic cells could be transfected with the pHBV1.3 plasmid, which caused the hepatitis B virus (HBV) genes to replicate in SV40LT-immortalized mouse hepatic cells. The expressions of hepatitis B surface antigen and hepatitis B c antigen, as well as the presence of HBV DNA replication intermediates, were observed in the pHBV1.3-transfected cells. This cell model will contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

Song XG, Bian PF, Yu SL, Zhao XH, Xu W, Bu XH, Li X, Ma LX. Expression of hepatitis B virus 1.3-fold genome plasmid in an SV40 T-antigen-immortalized mouse hepatic cell line. *World J Gastroenterol* 2013; 19(44): 8020-8027 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8020.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8020

INTRODUCTION

Chronic hepatitis B (CHB) is a severe public health problem that affects more than 400 million people worldwide and causes more than one million deaths annually[1]. Recent studies have shown that the correlation between serum hepatitis B virus (HBV) DNA levels and the risk of developing cirrhosis and hepatocellular carcinoma (HCC) is stronger than other baseline or virologic parameters[2]. The ultimate long-term goal of therapy is to achieve a “durable response” to prevent hepatic decompensation, reduce or prevent progression to cirrhosis and/or HCC, and prolong survival[3]. Moreover, it has now become clear that continuous suppression of HBV replication can revert liver fibrosis or even cirrhosis in most patients[4].

Treatment of hepatitis B depends on several factors, such as the stage of disease, the presence or absence of the “e” antigen, and the potential for drug resistance and subsequent inability to use a medicine, particularly in the advanced stages of chronic disease of the liver. Therefore, it is very important to evaluate these factors at the time the decision is made regarding the type and duration of treatment[5]. Interferon alpha-2a and interferon alfa-2b (IFN 2a and 2b)-based therapies have been used for many years as the preferential treatment approaches for cases with low levels of HBV DNA and high levels of alanine aminotransferase (ALT)[6-8]. The goal of treatment is to activate an immune response leading to hepatitis B e antigen (HBeAg) seroconversion[9]. This type of treatment, as the first option to modulate the immune system, aims to achieve elimination or remission.

However, this course of treatment results in a high cost, and also produces many adverse side effects, such as anaemia, a significant decrease in hemoglobin, vomiting, cold sweats and nausea[10]. Previous studies have reported that only about one out of three patients receives IFN therapy[11-13]. Furthermore, nucleoside analogues cannot completely eliminate the virus, and may lead to the mutation of the virus[14]. Thus, the development of new antiviral treatments remains a major research task. Additionally, new suitable HBV-infected animal cell models are urgently required to evaluate new treatment strategies. Therefore, this study aimed to establish a new immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression, and to investigate the expression of the HBV 1.3-fold genome plasmid (pHBV1.3) in the established SV40T-immortalized mouse hepatic cell line.

MATERIALS AND METHODS

Establishment of SV40T-immortalized mouse hepatic cell line

Kunming mice (3-5 d old) were provided by the clinical drug trial-based Animal Laboratory of Shandong University. Livers were collected from these mice, and mouse hepatic cells were isolated from the liver tissue fragments by the direct collagenase digestion method[15]. The isolated cells were cultured in the 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin and 100 mg/mL streptomycin, and placed in the incubator with 5% CO_2 and 37 ℃. All animals received humane care in compliance with the Principles of Laboratory Animal Care. The protocol was approved by the Animal Care and Use Committee.

The pRSV-T plasmid[16] was provided by Professor Reddel RR of the Australia Children’s Medical Research Institute. The pRSV-T plasmid was transfected into primary mouse hepatic cells according to the instructions of the liposome transfection kit (Invitrogen, Grand Island, United States). Twenty-four hours later, the 1640 culture medium with 10% FBS was added. Forty-eight hours later, it was replaced by the 1640 culture medium supplemented with 10% FBS and 500 μg/mL G418. Cells were passaged every 5 d at a ratio of 1:2.

An SV40 monoclonal antibody (Thermo,Waltham, United States) was used to detect the SV40T antigen and its distribution in SV40T-transfected cells by the indirect immunofluorescence assay. Primary mouse hepatic cells were employed as the negative control.

An inverted phase contrast microscope and an electron microscope were used to observe the morphology and ultrastructure of the SV40T-transfected mouse hepatic cells.

The supernatants of primary and immortalized mouse hepatic cell cultures were collected. The levels of ALT,

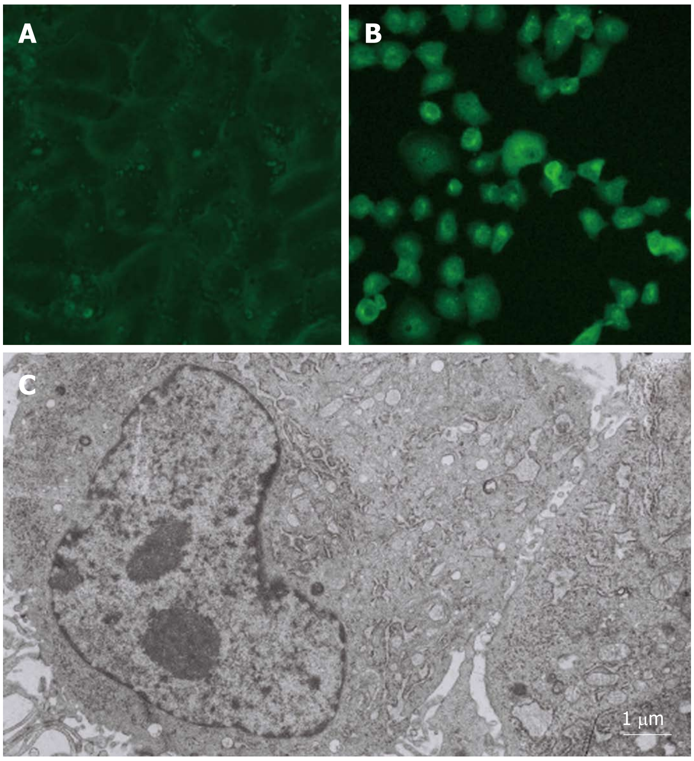

Figure 1 SV40 T-antigen-immortalized mouse hepatic cells (× 200). A: SV40 T-antigen (SV40T)-immortalized mouse hepatic cells visualized by an inverted phase contrast microscope; B: SV40T antigen immunofluorescence in mouse hepatic cells; C: SV40T-immortalized mouse hepatic cells visualized by an electron microscope.

aspartate aminotransferase (AST) and alpha-fetoprotein (AFP) were determined by an automatic biochemical analyzer (Beckman, Boulevard Brea, United States). Primary cultured mouse hepatic cells were employed as the control.

After total RNA extraction of primary and SV40T-transfected hepatic cells with an RNA extraction kit (Invitrogen), reverse transcription polymerase chain reaction (RT-PCR) was used to determine albumin (ALB) mRNA levels as previously described[17].

Western blotting was used to detect the presence of cytokeratin-18 (CK-18) in primary and SV40T-transfected mouse hepatic cells (22nd generation). Rabbit anti-mouse cell CK-18 was the primary antibody employed and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (IgG) was used as the secondary antibody. These two antibodies were purchased from Boster Biological Technology, Ltd (Wuhan, China).

Transfection

pHBV1.3[18] was provided by Professor Yin-Ping Lu of Huazhong University of Science and Technology. pHBV1.3 contains a 1.3-fold HBV genome (ayw subtype). Following the instructions of the Lipofectamine 2000 transfection kit (Invitrogen), the pHBV1.3 plasmid was transfected into SV40T-immortalized cells (22nd generation).

Electrochemiluminescence

The supernatants of the pHBV1.3-transfected cell cultures were collected at different times. An AXSYM automatic electrochemiluminescence immunoassay analyzer (Abbott) was used to quantify the levels of HBsAg and HBeAg.

Indirect immunofluorescence

pHBV1.3-transfected cells were seeded in 24-well plates. The cells were washed with PBS three times and fixed with 4% paraformaldehyde at 4 ℃ for 20 min. The cells were then washed with PBS three times again and incubated with PBS-diluted 10% goat serum for 30 min. Then the indirect immunofluorescence assay was performed using a fluorescence kit. Mouse anti-HBcAg and mouse anti-HBsAg were purchased from Millipore Corporation, while fluorescein Isothiocyanate-conjugated goat anti-mouse IgG was purchased from Southern Biotech (Birmingham, United States).

Southern hybridization and transmission electronic microscopy

At 72 h post-transfection, a DNA extraction of pHBV1.3-transfected cells was performed for Southern hybridization analysis. The probe was the digoxigenin-labeled 3.2 kb HBV DNA[19]. The Southern kit was purchased from Roche (Indianapolis, United States). Southern hybridization was carried out according to the manufacturer's instructions.

At 72 h after transfection, the supernatants of pHBV1.3-transfected cell cultures were collected. JEOL transmission electronic microscopy (JEM-1200EX Electron Microscope) was used to visualize the cells and photographs were taken.

Ethics statement

Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, Shandong University, and with the

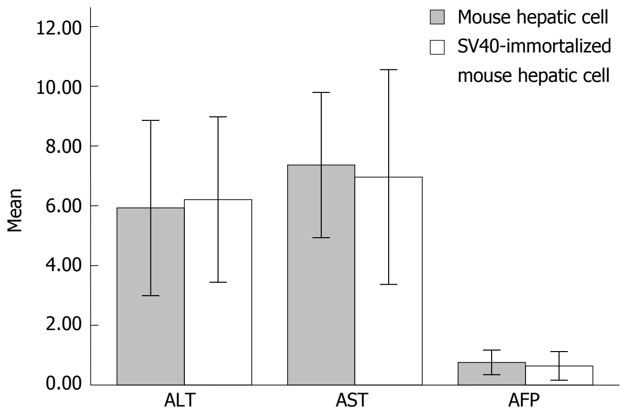

Figure 2 Levels of alanine aminotransferase, aspartate aminotransferase and alpha-fetoprotein in the cell culture supernatant. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: α-fetoprotein.

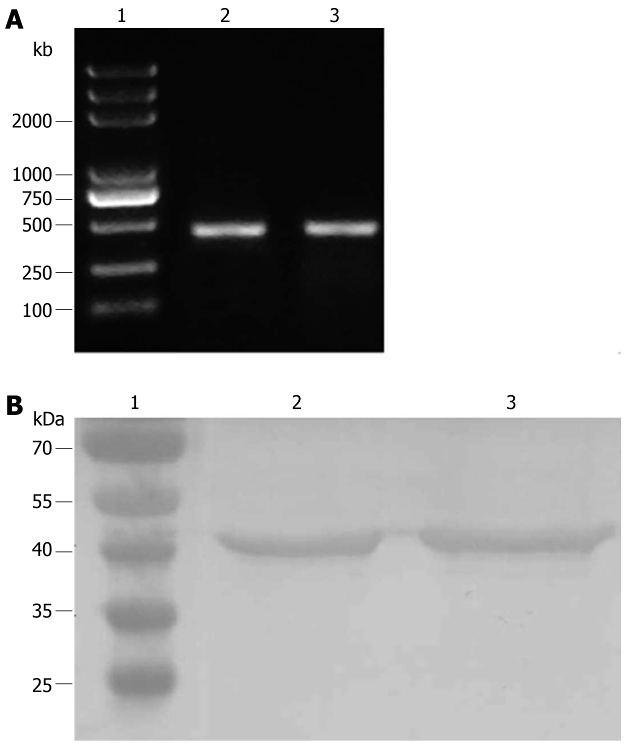

Figure 3 Electrophoresis and Western blotting. A: Electrophoresis of determine albumin (ALB) reverse transcription polymerase chain reaction products (1: markers; 2: primary mouse hepatic cells; 3: immortalized mouse hepatic cells at 22nd generation); B: ALB by Western blotting (1: Markers; 2: Primary mouse hepatic cells; 3: Transfected mouse hepatic cells at 22nd generation).

1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC, United States). The study was approved by the Institutional Animal Care and Use Committee at Shandong University (approval no. SDU003341201).

Statistical analysis

The data are presented as means ± SD. Comparisons between groups of data were performed using Student's *t* test. A difference with *P* value < 0.05 was considered to be statistically significant. Data were analyzed with the SPSS 11.0 statistical software package (SPSS Inc.; Chicago, IL, United States).

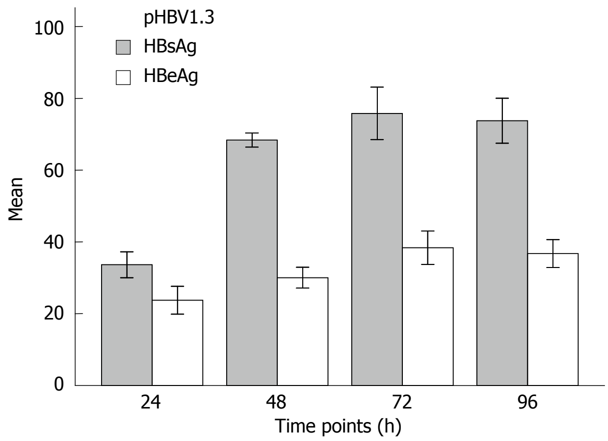

Figure 4 Levels of hepatitis B surface antigen and hepatitis B e antigen in the cell culture supernatant. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; pHBV1.3: Hepatitis B virus 1.3-fold genome plasmids.

RESULTS

Evaluation of SV40T-immortalized mouse hepatic cell line

The epithelial cell-like positive clones were found 30 d after the mouse hepatic cells were transfected with a SV40T-expressing plasmid (pRSV-T) by lipofection; these cells were an adherent monolayer and flat-shaped and presented in a polygonal, cluster-like multi-cell arrangement (Figure 1A). The SV40T mouse hepatic cells displayed the typical morphology and structure of hepatic cells, and many glycogen granules, mitochondria and endoplasmic reticulum structures were clearly visible under the electron microscope (Figure 1C). Furthermore, the splitting dual-core cells reflected the *in vitro* proliferation and differentiation processes of the transfected hepatic cells (Figure 1C). Cells were passaged every five d at a ratio of 1:2 for 38 generations, and no change in cell morphology was observed.

After SV40T transfection, the SV40 T-antigen immunofluorescence of the mouse hepatic cells gradually increased, and was visible 30 d after transfection. Matte-like fluorescence could be clearly detected in the cytoplasm, along with granular-like fluorescence in the nucleus (Figure 1B).

The quantified levels of ALT, AST and AFP in the supernatant of the cultures are shown in Figure 2. The levels of ALT, AST and AFP in the supernatant of mouse hepatic cell and SV40T-transfected hepatic cell cultures were 5.93 ± 1.47 *vs* 6.21 ± 1.38 (t = 0.481, P = 0.636), 7.36 ± 1.21 *vs* 6.96 ± 1.79 (t = 0.643, P = 0.527) and 0.76 ± 0.21 *vs* 0.65 ± 0.24 (t = 1.318, P = 0.201), respectively (n = 12). No significant difference in the levels of ALT, AST and AFP was observed between the mouse hepatic cell and SV40T-transfected hepatic cell cultures (*P*

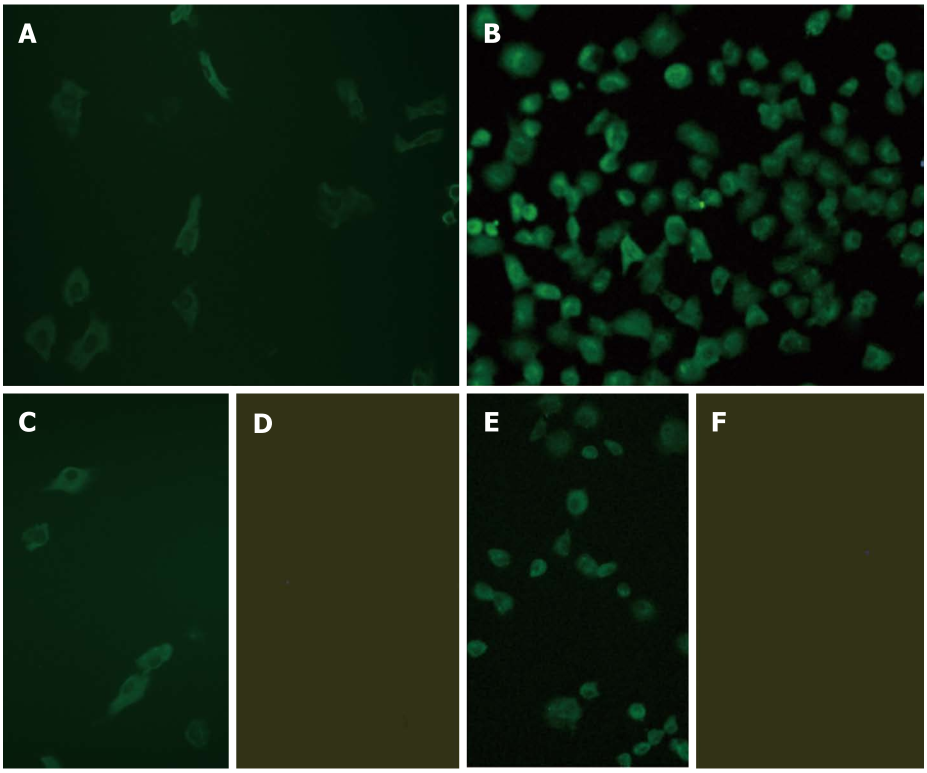

Figure 5 Analysis of hepatitis B surface antigen and hepatitis B c antigen expression in hepatitis B virus 1.3-fold genome plasmids-transfected cells by immunofluorescence microscopy (× 200). A: Hepatitis B surface antigen (HBsAg) observed in the hepatitis B virus 1.3-fold genome plasmids (pHBV1.3)- transfected cells at 24 h post-transfection; B: Hepatitis B c antigen (HBcAg) observed in the pHBV1.3-transfected cells at 24 h post-transfection; C: HBsAg observed in HepG2.215 cells (positive control); D: HBsAg observed in untransfected SV40 T-antigen (SV40T)-immortalized cells (negative control); E: HBcAg observed in HepG2.215 cells (positive control); F: HBcAg observed in untransfected SV40T-immortalized cells (negative control).

> 0.05).

Following the total RNA extraction of SV40T-transfected hepatic cells (22nd generation) and RT-PCR, the ALB mRNA was apparent as a bright band at 475 bp (Figure 3A), indicating that SV40T-immortalized mouse hepatic cells had the ability to express ALB mRNA. Mouse hepatic cells were employed as the positive control.

Following the protein extraction of SV40T-transfected hepatic cells (22 generation), SDS-PAGE and Western blotting were carried out. Immunoblotting of SV40T-transfected hepatic cells demonstrated their expression of CK-18, and mouse hepatic cells, employed as the positive control, also displayed immunoreactivity for CK-18, as expected (Figure 3B).

Expression of pHBV1.3 in SV40T-immortalized mouse hepatic cells

The levels of HBsAg and HBeAg in the supernatant were monitored 24, 48, 72 and 96 h after pHBV1.3 transfection. The results of this analysis are shown in Figure 4. The levels of HBsAg and HBeAg in the supernatant continuously increased after transfection of pHBV1.3, though they both began to gradually decrease after 72 h.

The expression of HBsAg and HBcAg were observed in the pHBV1.3-transfected cells at 24 h after transfection by immunofluorescence microscopy, with expression reaching a peak at 72 h. HBsAg was mainly observed in the cytoplasm, while HBcAg was detected in both the cytoplasm and nucleus, especially in the former (Figure 5). HBV DNA replication intermediates, including rcDNA, dsDNA and ssDNA, were also observed 72 h after transfection (Figure 6). Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were observed in the supernatant (Figure 6).

DISCUSSION

The development of another HBV *in vitro* cell model would provide a very important tool to study the biological characteristics of HBV, the pathogenesis of hepatitis B, the mechanism of carcinogenesis by HBV infection, and carry out *in vitro* anti-HBV drug screening 2.2.15 cells, a stable cell line which harbors the HBV genome, and can support HBV replication and the secretion of infectious virus particles[20-23]. However, the low expression of HBV was due to the low viral genome copy number integrated in the host cell chromosome. This drawback limits the application of this cell line as an *in vitro* cell model of HBV infection, especially as an infection model for antiviral drug-resistant mutant screening and investigation of biological characteristics[24].

Simian vacuolating virus 40 (SV40), which was first obtained from cultured rhesus monkey kidney cells[25], belongs to the *Papovaviridae* family[26]. The transfection of

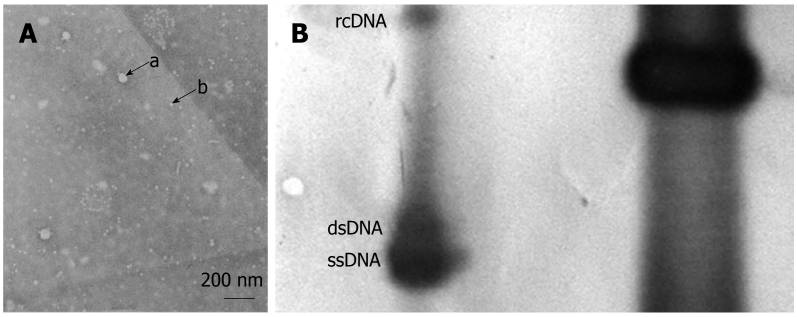

Figure 6 Expression of hepatitis B virus 1.3-fold genome plasmids in SV40 T-antigen-immortalized mouse hepatic cells. A: 42 nm Dane particles (a) and 22 nm subviral particles (b) in the supernatant; B: Hepatitis B virus (HBV) DNA replication intermediates in HBV 1.3-fold genome plasmids (pHBV1.3)-transfected cells 24 h after transfection. rc DNA: Relaxed circular DNA; dsDNA: Double-stranded DNA; ssDNA: Single-stranded DNA.

the SV40 early gene into cells is the most common method for cell immortalization. There are different opinions regarding the mechanism by which SV40LT causes cell immortalization[27,28]. This method has been found to be useful for a variety of human cell types[29]. The use of SV40 for cell immortalization has a long history. It has been used to immortalize epithelial cells of the bronchial intrahepatic bile duct, cervical cells and other cells[30-32]. Many studies of SV40T-immortalized cell lines have shown that the transduction of the *SV40T* gene can increase the growth rate of cells, while retaining the differentiation phenotype of many original cells and keeping the biological characteristics of the original cells, with the rare expression of a non-original gene[33,34]. These characteristics make the SV40T-immortalized cells suitable for use as an *in vitro* model. In this study, the pRSV-T plasmid was used to transfect the mouse hepatic cells to establish an SV40T-immortalized mouse hepatic cell line, which was selected through 500 μg/mL G418 screening.

Our experimental results showed that there were no significant differences in morphology between primary and SV40T-immortalized mouse hepatic cells. Furthermore, the same level of expression and secretion of ALB, as well as the CK-18 activity, was observed between the primary and SV40T-immortalized mouse hepatic cells (without any tumorigenic potential). At present, the SV40T-immortalized mouse hepatic cells have been passaged to the 38th generation.

The genome of the HBV 1.3-fold genome plasmid is smaller than that of the HBV 2.0-fold plasmid, and its efficiency of replication and expression is higher than that of the 1.2 and 1.1-fold plasmids[35]. The HBV 1.3-fold genome plasmid contains HBV 5'-end Enh I, Enh II, replication-origin (DR1, DR2), the former genome transcription start site, x and pre-C promoter, and x open reading frame. Hence, the HBV 1.3-fold genome plasmid is used most frequently. After Huh7 and HepG2 cells were transfected with the recombinant plasmid pHBV1.3, the *in vitro* replication and expression of the *HBV* gene could be detected, and high levels of HBsAg/HBeAg intermediates and transcripts involved in HBV DNA replication were also found[18,36]. In this study, the pHBV1.3 plasmid was transfected into SV40T-immortalized mouse hepatic cells. After transfection, the expression of HBsAg and HBcAg was observed in the pHBV1.3-transfected cells. Additionally, HBV DNA replication intermediates, including relaxed circular DNA, double-stranded DNA and single-stranded DNA, were also observed 72 h after transfection. Furthermore, a few 42 nm Dane particles, as well as many 22 nm subviral particles with a spherical or filamentous shape, were observed in the supernatant.

In summary, our findings suggest that the expression of the *SV40T* gene immortalized a mouse hepatic cell line, and subsequent transfection of pHBV1.3 established a new *in vitro* cell model for anti-HBV drug research.

COMMENTS

Background

Chronic hepatitis B (CHB) is a severe public health problem, and treatment of hepatitis B poses many challenges, such as treatment for an advanced stage of disease and the potential for drug resistance. Thus, the development of new antiviral treatments remains a major research task, and new suitable hepatitis B virus (HBV)-infected *in vitro* cell models are urgently required to evaluate new therapeutic strategies. With this in mind, this study aimed to establish a new immortalized mouse hepatic cell line induced by SV40 T-antigen (SV40T) expression, and to investigate the consequences of HBV 1.3-fold genome plasmid (pHBV1.3) expression in this SV40T-immortalized mouse hepatic cell line.

Research frontiers

SV40LT antigen has the ability to immortalize some animal cells. The genome of the HBV 1.3-fold genome plasmid is smaller than that of the HBV 2.0-fold plasmid, and its efficiency of replication and expression is higher than that of the 1.2- and 1.1-fold plasmids. The HBV 1.3-fold genome plasmid contains HBV 5'-end Enh I, Enh II, replication-origin (DR1, DR2), the former genome transcription start site, x and pre-C promoter, and x open reading frame. Hence, the HBV 1.3-fold genome plasmid is used most frequently. The genome of the HBV 1.3-fold genome plasmid has been shown to replicate in HepG2 and Hu7 cell lines. According to the authors, there has been no report concerning the expression of the HBV 1.3-fold genome plasmid in an immortalized mouse hepatic cell line.

Innovations and breakthroughs

The SV40LT antigen immortalized mouse hepatic cells, which was not found in previous reports. In this study, a new immortalized mouse hepatic cell line was established through the transfection of the pRSV-T plasmid into primary mouse hepatic cells. The genome of the HBV 1.3-fold genome plasmid can replicate in human hepatoma cell lines (HepG2 or HUH7 cells). The authors successfully transfected the pHBV1.3 plasmid into the immortalized mouse hepatic cell line and observed the expression of HBV. The new cell model established in this study will contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

Applications

This cell model will contribute to the research of HBV and the evaluation of antiviral drugs *in vivo*.

Peer review

This is a good basic study in which authors transfected the pRSV-T plasmid into primary mouse hepatic cells and established a new immortalized mouse hepatic cell line. The authors transfected the pHBV1.3 plasmid into the immortalized mouse hepatic cell line and observed the expression of HBV. This cell model would contribute to the research of HBV and the evaluation of anti-viral drugs *in vivo*.

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BRIEF ARTICLE

Evaluation of 4 three-dimensional representation algorithms in capsule endoscopy images

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Abstract

AIM: To evaluate the three-dimensional (3-D) representation performance of 4 publicly available Shape-from-Shading (SfS) algorithms in small-bowel capsule endoscopy (SBCE).

METHODS: SfS techniques recover the shape of objects using the gradual variation of shading. There are 4 publicly available SfS algorithms. To the best of our knowledge, no comparative study with images obtained during clinical SBCE has been performed to date. Three experienced reviewers were asked to evaluate 54 two-dimensional (2-D) images (categories: protrusion/inflammation/vascular) transformed to 3-D by the aforementioned SfS 3-D algorithms. The best algorithm was selected and inter-rater agreement was calculated.

RESULTS: Four publicly available SfS algorithms were compared. Tsai's SfS algorithm outperformed the rest (selected as best performing in 45/54 SBCE images), followed by Ciuti's algorithm (best performing in 7/54 images) and Torreão's (in 1/54 images). In 26/54 images; Tsai's algorithm was unanimously selected as the best performing 3-D representation SfS software. Tsai's 3-D algorithm superiority was independent of lesion category (protrusion/inflammatory/vascular; *P* = 0.678) and/or CE system used to obtain the 2-D images (MiroCam®/PillCam®; *P* = 0.558). Lastly, the inter-observer agreement was good (*kappa* = 0.55).

CONCLUSION: 3-D representation software offers a plausible alternative for 3-D representation of conventional capsule endoscopy images (until optics technology matures enough to allow hardware enabled-"real" 3-D reconstruction of the gastrointestinal tract).

Key words: Capsule endoscopy; Small-bowel; Three-dimensional; Software; Algorithm; Reconstruction; Technology; Advance

Core tip: Accurate three-dimensional (3-D) reconstruction of the gastrointestinal tract requires the use of stereo-cameras that can simulate human binocular vision. In the absence of such technology in capsule endoscopy, we rely on software approaches [such as the Shape-from-Shading (SfS) algorithms] to obtain 3-D representation of digestive tract structures. In the present study, we evaluated the use of 4 publically available SfS in capsule endoscopy. 3 experienced/experts reviewers concluded that Tsai's approach is the best of the four available algorithms.

Karargyris A, Rondonotti E, Mandelli G, Koulaouzidis A.

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INTRODUCTION

Capsule endoscopy (CE) has changed our diagnostic approach for small-bowel diseases[1,2]. Although more accurate and of higher diagnostic yield than other modalities[3,4], there are still occasions where pathology is either missed or misinterpreted[5-7]. Furthermore, reports have shown that three-dimensional (3-D) reconstruction can facilitate diagnosis by enhancing textural features of mucosal structures or intestinal abnormalities[8,9]. However, accurate 3-D reconstruction of the gastrointestinal (GI) tract requires the use of stereoscopic cameras that can simulate human binocular vision[10,11]. With the current level of technological investment in CE though *i.e.*, camera size, packaging constraints and power consumption, accurate 3-D imaging of the intestinal lumen in small-bowel capsule endoscopy (SBCE) is still unfeasible[9,12].

Therefore, software approaches that offer 3-D representation of conventional monocular two-dimensional (2-D) CE frames have been developed[13] and proposed for use in CE[14]. Such approaches *e.g.*, Shape-from-Shading (SfS) algorithms, are members of a family of shape recovery algorithms called shape-from-X techniques (Figure 1)[13]. Given a single 2-D image, these algorithms recover the shape of objects using the gradual variation of shading[13]. Essentially, surface "reconstruction" with SfS is achieved through a mathematical representation that is inverted in order to recover dense surface distance and normal information by the gradual variation of shading[13]. We were able to retrieve 4 publicly available SfS algorithms[15-18]. To the best of our knowledge, no comparative study with images obtained during clinical SBCE has been performed to date[19]. We aimed to evaluate the 3-D representation performance of 4 publicly available SfS algorithms by comparing them with their equivalent 2-D images of small-bowel structures/lesions obtained during SBCE, in order to identify the algorithm more helpful in facilitating identification and distinction between lesion and surrounding mucosa.

MATERIALS AND METHODS

Between January 2011 and January 2012, 262 SBCE procedures were performed at the Royal Infirmary of Edinburgh (tertiary referral centre for CE for the southeast of Scotland, United Kingdom) in 249 patients (mean age: 52.6 ± 12.1 years), as already described elsewhere[9]. Out of them, 140 were performed with PillCam®SB2 (Given ®Imaging Ltd., Yokneam, Israel) and 122 with MiroCam® (IntroMedic®Co, Seoul, South Korea). A total of 54 were selected images (27 obtained with MiroCam® and 27 with PillCam®SB) on the basis of the overall quality *i.e.*, brightness, absence of air bubbles, debris, or opaque luminal fluid and clarity of findings (lesions or structures). Thereafter, images were classified in the following image groups: (1) vascular lesions *i.e.*, angioectasias (n = 16); (2) inflammatory lesions *i.e.*, ulcers, erosions, aphthae, cobblestone, fold and/or villous oedema (n = 18); and (3) protruding lesions/structures *i.e.*, polyp/mass, nodular lymphoid hyperplasia, cluster of focal lymphangiectasia, chylous cysts, and ampulla of Vater, (n = 20).

3-D image representation software

All selected images were reconstructed in 3-D by means of all 4 SfS algorithms. Three reviewers (Rondonotti E, Mandelli G, Koulaouzidis A) with extensive CE experience and blinded to each other participated in this study. In order to facilitate the evaluation process, a Mathworks© Matlab program with a graphic user interface (GUI) was developed (Figure 2; a video presenting the evaluation process is provided as supplementary material *via* this link: https://dl.dropboxusercontent.com/u/7591304/EvaluationVideo.mov). The program consisted of two windows in which the conventional 2-D SBCE image (Figure 2, single frame at the right side/window of the GUI screen) and its corresponding 3-D represented images (four, one for each of the 4 SfS under evaluation) are presented to the reviewer (Figure 2, left side/window of the GUI screen).

The 3-D SfS representations appeared in random order. The reviewers had the ability and freedom to rotate and zoom in each of the 3-D represented images. At the bottom of the GUI screen, a single "task request": "Choose the 3-D representation you consider most helpful in distinguishing the finding (seen in 2-D) from the surrounding mucosa" appeared. This prompted reviewers to choose one among the four 3-D 'reconstructed' images, each generated by a different 3-D algorithm. After selecting the best SfS representation, the reviewer had to click "next" to proceed to the next case. This process was repeated until the program reached the last case after which each separate evaluation was concluded.

Outcome measures

Reviewers were asked to evaluate 54 images. The following subgroup analyses were performed: (1) evaluation of 3-D representation according to the type of finding (vascular *vs* inflammatory *vs* protruding); and (2) evaluation according to the system generating the 2-D image (PillCam® *vs* Mirocam®). Furthermore, inter-observer agreement was calculated.

Ethics consideration

This study was conducted in accordance with United Kingdom research ethics guidelines. After review by the local ethics committee further specific ethical review and approval were not required, as the study was considered an evaluation of previously collected endoscopy images, using data already obtained as part of regular clinical care[20].

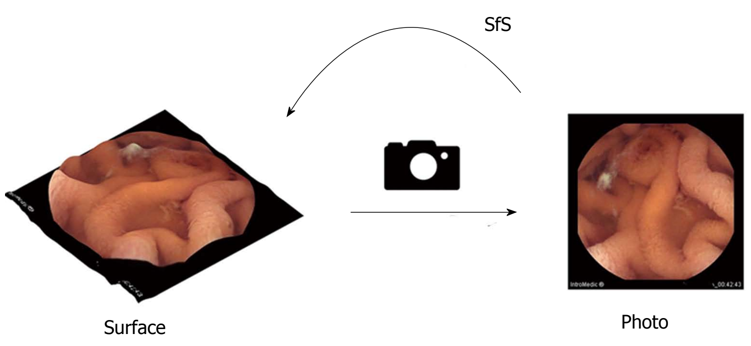

Figure 1 Shape-from-Shading function. Capturing a surface using a camera removes depth information. Shape-from-Shading (SfS) techniques try to reproduce the missing depth information from a given two-dimensional (2-D) image.

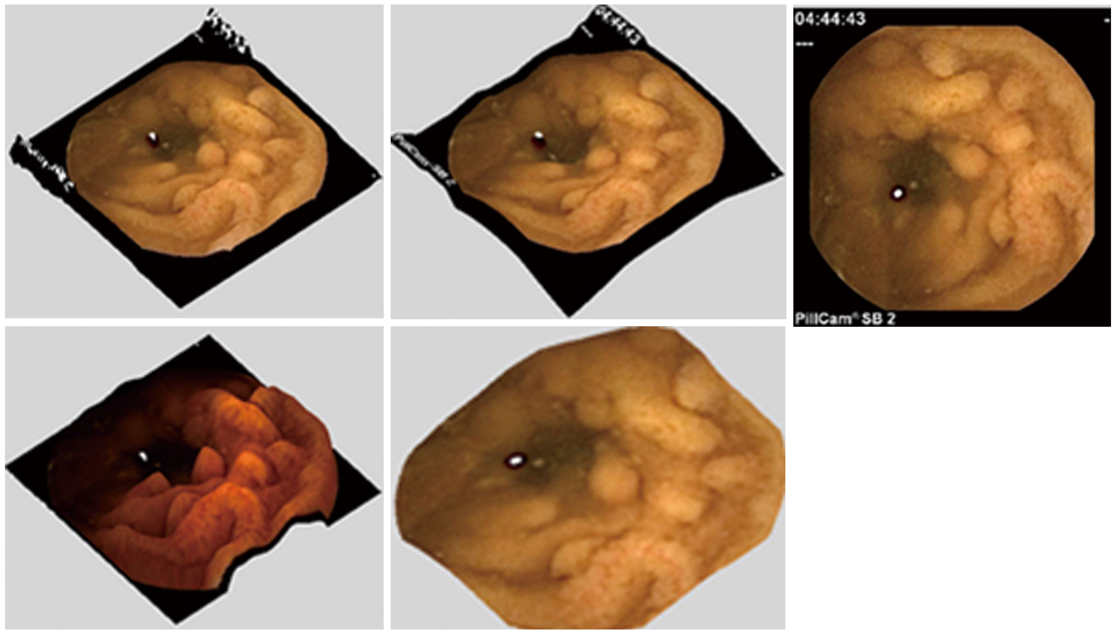

Figure 2 For the evaluation phase, a Mathworks© Matlab program with a graphic user interface was developed. The program consists of two windows in which the conventional two-dimensional capsule endoscopy image (single frame at the right side/window of the graphic user interface screen) and its corresponding three-dimensional represented images (four, one for each of the 4 shape-from-shading under evaluation) were presented to the reviewer.

Table 1 Results of the Shape-from-Shading method per lesion category

SfS method	Vascular		Inflammatory		Protrusion	
	PillCam®	MiroCam®	PillCam®	MiroCam®	PillCam®	MiroCam®
Tsai	7	7	7	6	8	10
Ciuti	1	0	1	0	1	4
Torreão	0	0	1	0	0	0
Barron	0	0	0	0	0	0
None selected	0	1	0	0	0	0

SfS: Shape-from-Shading.

Statistical analysis

For numerical variables, values are presented as mean ± SD. Where necessary, the Fisher exact test was calculated. A two-tailed *P* value < 0.05 was considered statistically significant. Inter-observer agreement was calculated using an online *kappa* calculator (available from http://justus-randolph.net/kappa/) which provides the calculation of Randolph's free-marginal multirater *kappa* [21], applicable when raters are not forced to assign a certain number of cases to each category. Values of *kappa* can range from -1.0 to 1.0, with -1.0 indicating perfect disagreement below chance, 0.0 indicating agreement equal to chance, and 1.0 indicating perfect agreement above chance. More specifically, the inte is classified per *kappa* as poor < 0.20, fair 0.2-0.40, good 0.41-0.60, very good 0.61-0.80 and, excellent 0.81-1.00[22]. All other statistical analyses were performed using a statistical package, StatsDirect, StatsDirect Ltd, Altrincham, Cheshire, United Kingdom.

RESULTS

Of the 4 SfS algorithms, Tsai's 3-D algorithm outperformed the rest (selected as best in 45/54 images), followed by Ciuti's (best performing SfS in 7/54 images) and Torreão's (in 1/54 images); there was a single image for which each reviewer selected (as best performing) a different 3-D representation algorithm. Of note, not once was Barron's 3-D algorithm selected as best performing (Table 1, Figure 3).

In 26/54 images, Tsai's algorithm was unanimously

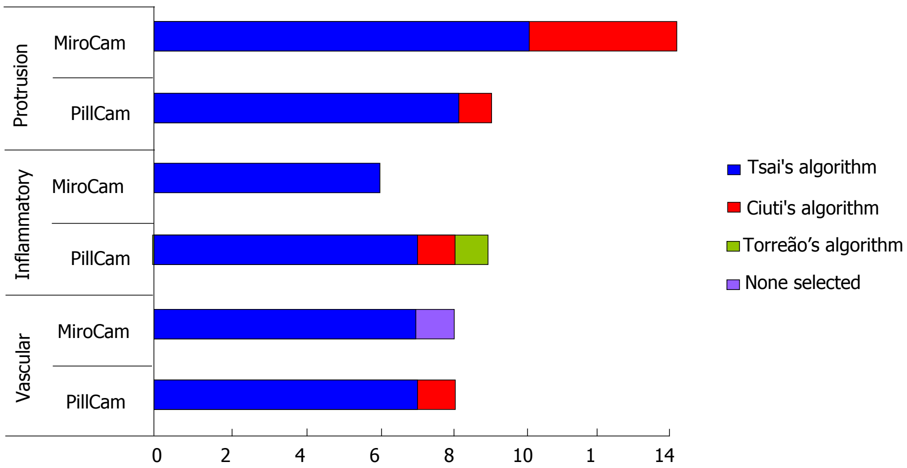

Figure 3 Assessment results for the 4 Shape-from-Shading algorithms per lesion category.

selected as the best performing 3-D representation SfS software. Tsai's 3-D algorithm superiority was independent of lesion category (protrusion/inflammatory/vascular; P = 0.678) and/or CE system used to obtain the 2-D images (MiroCam®/PillCam®; P = 0.558). Lastly, the inter-observer agreement was good (*kappa* = 0.55).

DISCUSSION

In the present study, we compared the performance of 4 publicly available 3-D "reconstruction" algorithms[15-18] (SfS software) using 54 conventional 2-D CE images. The evaluation criterion was subjective *i.e.*, perceived visualisation improvement (3-D representations offered over the corresponding conventional 2-D images) by 3 experienced CE reviewers. Based on this evaluation, Tsai's algorithm is the 3-D representation model recommended for use in CE. This outcome directly supports Tsai's SfS model theoretical advantages: (1) able to produce good results for round surfaces, which are the case for most digestive tract shapes; and (2) it behaves quite well with bright surfaces[13].

Depth information is an important aspect of human vision; it helps human brain to analyse and comprehend the surrounding environment. Images captured with conventional (non-stereoscopic) cameras "discard" the 3rd dimension (depth) as conventional cameras can only save 2 dimensions (height and width). Therefore depth information is lost; and moreover, most imaging algorithms perform less efficiently.

To date, engineers have not been able to equip capsule endoscopes with stereoscopic cameras for the following reasons: (1) packaging/space limitations; (2) low depth resolution of stereoscopic or time-of-flight cameras[22-24]; and (3) power consumption issues. However, it is almost certain that in the foreseeable future these hardware-related limitations will be overcome[11] and eventually 3-D CE will be a commodity. Nevertheless, until hardware changes are widely implemented, several efforts have been made to convert 2-D images into 3-D images (3-D representation or "reconstruction") through software and dedicated algorithms. There are software algorithms that offer a fair trade-off between 2-D images and hardware-enabled 3-D images. These algorithms are part of a family of shape recovery algorithms called Shape-from-X techniques[13]. Basically a SfS algorithm recovers the shape of objects, given a single monocular image, using the gradual variation of shading[8,13].

SfS algorithms can be divided into four groups: (1) minimization approaches[16-18]; (2) propagation approaches; (3) local approaches; and (4) linear approaches[15]. It is important to remember that each of the 4 SfS algorithms evaluated herein utilizes a different approach to recover the shape from a conventional 2-D image.

More specifically, Tsai *et al*[15] described an repetitive update of the depth using a linear approximation of the reflectance function. Ciuti *et al*[16] used a camera model with perspective projection and a light source close to the surface and away from the optical centre to measure depth. Torreão *et al*[17] applied a linear-nonlinear biological model that mimics neuronal responses to estimate shape. Finally, Barron *et al*[18] proposed a unified model for recovering shape, reflectance and optional illumination while using local smoothness, global scarcity or entropy, and the absolute colour of each pixel. Although Tsai's[14,15] method is very straightforward and to an extent simplistic, it provides satisfying results. Cuiti's *et al*[16] algorithm, on the other hand, uses a more advanced model (incorporating a camera model with perspective projection) that makes things in the background appear further back than in Tsai's model (Figure 4).

Since for a given 2-D image, light source and surface shape are not known, these algorithms try to model how the 2-D image was created from the 3-D environment to finally produce an approximation this 3-D depth. The above modelling has a significant impact on the resulting 3-D representation. During SfS process additional constraints need to be applied on the surface shape parameters or the light conditions to find the surface characteristics.

In conclusion, we showed previously that 3-D representation software offers a plausible alternative for 3-D representation of conventional CE images (until optics technology matures enough to allow a hardware enabled-"real" 3-D reconstruction of the GI tract)[9]. In the pres-

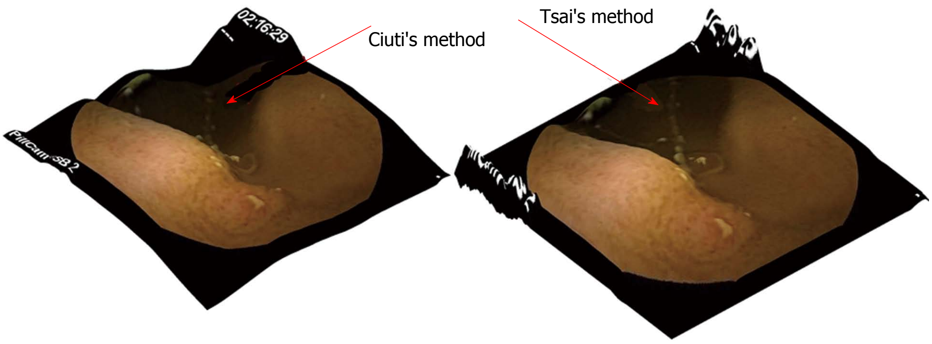

Figure 4 Ciuti's algorithm (left) and Tsai's method (right). Although Tsai's method is very straightforward and to an extent simplistic, it provides satisfying results. Ciuti's *et al*[16] algorithm, on the other hand, uses a more advanced model that makes things in the background appear darker than in Tsai's model.

ent study we compared 4 publicly available SfS methods. 3-D reconstruction is attracting interest in capsule endoscopy[8,9,14,25-28], especially as newly developed and/or under development CE become available, with greater potential (due to imager and optics) for 3-D software[20].

COMMENTS

Background

Over the past decade, conventional endoscope technology has advanced with the use of three-dimensional (3-D) cameras offering increased diagnostic and interventional capabilities. Unfortunately, due to hardware limitations, 3-D small-bowel capsule endoscopy (SBCE) is still an open technological challenge. It is aspired that 3-D SBCE will be able to offer similar benefits to conventional 3-D endoscopy. Therefore, information technology engineers suggested the use of software techniques (Shape-from-Shading, SfS) methods that simulate 3-D reconstruction *i.e.*, 3-D representation in SBCE images. To date, various SfS approaches have been proposed; each aims to retrieve depth information from 2-D images (shape recovery) through different mathematical transformations, hence offering different shape approximations.

Research frontiers

The authors aimed to evaluate the 3-D representation performance of 4 publicly available SfS algorithms by comparing them with their equivalent 2-D images of small-bowel structures/lesions obtained during SBCE, in order to identify the algorithm more helpful in facilitating identification and distinction between the lesion and the surrounding mucosa.

Innovations and breakthroughs

This study, in conjunction with further similar work in the field, is useful in the assessing the potential validity of integrating 3-D representation in capsule endoscopy reviewing software.

Applications

Software-enabled 3-D representation is a promising approach that enables 3-D imaging at no additional cost. The authors have shown that SfS application leads to improved visualisation in SBCE and is it likely to be of use in certain clinical scenarios, like the 'mass or bulge' question.

Peer review

An interesting paper dealing with software and capsule endoscopy.

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BRIEF ARTICLE

Predictors of *Clostridium difficile* infection severity in patients hospitalised in medical intensive care

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Abstract

AIM: To describe and analyse factors associated with *Clostridium difficile* infection (CDI) severity in hospitalised medical intensive care unit patients.

METHODS: We performed a retrospective cohort study of 40 patients with CDI in a medical intensive care unit (MICU) at a French university hospital. We include patients hospitalised between January 1, 2007 and December 31, 2011. Data on demographics characteristics, past medical history, CDI description was collected. Exposure to risk factors associated with CDI within 8 wk before CDI was recorded, including previous hospitalisation, nursing home residency, antibiotics, antisecretory drugs, and surgical procedures.

RESULTS: All included cases had their first episode of CDI. The mean incidence rate was 12.94 cases/1000 admitted patients, and 14.93, 8.52, 13.24, 19.70, and 8.31 respectively per 1000 admitted patients annually from 2007 to 2011. Median age was 62.9 [interquartile range (IQR) 55.4-72.40] years, and 13 (32.5%) were women. Median length of MICU stay was 14.0 d (IQR 5.0-22.8). In addition to diarrhoea, the clinical symptoms of CDI were fever (> 38 ℃) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. The duration of diarrhoea was 13.0 (8.0-19.5) d. In addition to diarrhoea, the clinical symptoms of CDI were fever (> 38 ℃) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. Prior to CDI, 38 patients (95.0%) were exposed to antibiotics, and 12 (30%) received at least 4 antibiotics. Fluoroquinolones, 3rd generation cephalosporins, coamoxiclav and tazocillin were prescribed most frequently (65%, 55%, 40% and 37.5%, respectively). The majority of cases were hospital-acquired (n = 36, 90%), with 5 cases (13.9%) being MICU-acquired. Fifteen patients had severe CDI. The crude mortality rate within 30 d after diagnosis was 40% (n = 16), with 9 deaths (9 over 16; 56.3%) related to CDI. Of our 40 patients, 15 (37.5%) had severe CDI. Multivariate logistic regression showed that male gender [odds ratio (OR): 8.45; 95%CI: 1.06-67.16, P = 0.044], rising serum C-reactive protein levels (OR = 1.11; 95%CI: 1.02-1.21, P = 0.021), and previous exposure to fluoroquinolones (OR = 9.29; 95%CI:

1.16-74.284, *P* = 0.036) were independently associated with severe CDI.

CONCLUSION: We report predictors of severe CDI not dependent on time of assessment. Such factors could help in the development of a quantitative score in ICU's patients.

Key words: *Clostridium difficile*; Health-care associated infection; Hospital-acquired infection; Intensive care unit; Nosocomial infection; Severe *Clostridium difficile* infection

Core tip: We reported that male gender, rising serum C-reactive protein level, and previous exposure to fluoroquinolones were independently associated with severe *Clostridium difficile* infection (CDI) in medical intensive care unit. This could help in the development of a quantitative severity score that could fuel comparative effectiveness studies and prospective trials of CDI therapy in critically-ill patients.

Khanafer N, Touré A, Chambrier C, Cour M, Reverdy ME, Argaud L, Vanhems P. Predictors of *Clostridium difficile* infection severity in patients hospitalised in medical intensive care. *World J Gastroenterol* 2013; 19(44): 8034-8041 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8034.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8034

INTRODUCTION

Clostridium difficile (*C. difficile*) infection (CDI) has become a growing cause of nosocomial morbidity, high hospital costs and mortality in North America as well as in other areas of the world[1-6]. Hospital-acquired CDI has surpassed methicillin-resistant *Staphylococcus aureus* (*S. aureus*) in some hospitals as the leading source of healthcare-associated infections[7] and was ranked in the five most important scientific issues facing healthcare epidemiology[8]. Several mechanisms have been postulated to increase disease severity, including the emergence of specific strains with genetic polymorphisms that encode higher levels of bacterial toxins A and B as well as the production of a binary toxin[9-11]. Advanced age, severe co-morbidity, hospitalisation, antibiotic exposure, immunosuppressants and treatment with motility-influencing or acid-suppressive drugs have all been implicated as risk factors for CDI[12-17].

The cumulative mortality attributable to CDI for all patients typically ranges from 5.5% to 6.9% but can reach 16.7% during severe outbreaks[18-24]. In the United States, *C. difficile* is now the 9th leading gastrointestinal cause of death[25]. CDI is more common in the intensive care unit (ICU) setting, with an overall incidence of roughly 4%[26]. Up to 20% of ICU patients who develop symptomatic disease will progress to fulminant colitis with a mortality rate of nearly 60%[26]. In the United States, attributable costs range from $2871 to $4846 per case of primary CDI and from $13655 to $18067 for infection recurrence or relapse[18], with annual expenditures in excess of $3 billion[27]. A study of ICU patients disclosed gross costs of $11353 for CDI compared to $6028 without CDI[26].

CDI among critically-ill patients usually presents as diarrhoea, abdominal pain, hypotension, electrolyte perturbations, and fever[2,3,10,21,28,29]. Several studies have examined factors related to CDI acquisition and mortality in different medical units[30,31]. To the best of our knowledge, factors associated with CDI severity in medical ICUs (MICU) are poorly documented. We undertook a one-center cohort investigation to analyse factors linked with CDI severity and to report the prognosis of CDI in hospitalised MICU patients.

MATERIALS AND METHODS

Study population

This retrospective cohort study was performed at an 860-bed university-affiliated public hospital in Lyon, France. All adult patients with CDI diagnosed in a MICU (15 beds) between January 1, 2007 and December 31, 2011 were included. Patients were followed up until the last point of hospital contact. Thirty-day in-patient mortality from any cause was chosen as the primary endpoint. According to French law, a study like this one does not require ethics committee approval because it is observational and derives from a surveillance database approved under national regulations (*Comité National Informatique et Liberté*)[32]. Protocol design was approved by the hospital's institutional review board.

Data collection

After case identification, full medical files were reviewed and data collected through our institution's electronic medical database. The following data were analysed: age, sex, body weight, diagnosis on admission, co-morbidities, Glasgow coma score available on the day of ICU admission, nutritional status, parenteral nutrition administration, CDI symptoms and their duration, prior CDI history, results of microbiological tests, specific antibiotherapy for CDI, and evolution of infection. Exposure to risk factors associated with CDI within 8 wk before CDI was recorded, including previous hospitalisation, nursing home residency, antibiotics, antisecretory drugs (proton pump inhibitors, PPI), and surgical procedures (endoscopy, percutaneous gastrostomy, nasogastric feeding, gastrointestinal surgery). Leukocyte count, C-reactive protein, and serum albumin values were collected on days -2 to +2 relative to day 0 (the day the diarrhoeal sample was tested for CDI). Patient outcomes were analysed until in-patient death or last point of hospital contact.

Microbiological data

C. difficile testing was performed only on unformed stool samples from patients clinically suspected to have

CDI. Laboratory diagnosis of CDI was based on stool enzyme-linked immunosorbent assay (ELISA, Immuno-Card Toxins A and B, Meridian Biosciences, Cincinnati, OH, United States, Ref. 716060) coupled with toxigenic culture.

Definitions

All definitions were selected as part of routine CDI surveillance. Bacteriological cases of CDI were defined as positive enzyme linked immunosorbent assay (ELISA) results and/or positive toxigenic culture. Clinical CDI severity was considered when patients met at least 1 of the following criteria: endoscopically- or histologically-proven colitis or CDI-related complications, such as toxic megacolon, intestinal perforation, colectomy, septic shock, CDI requiring admission to ICU or related death in 30 d. It should be noted, however, that there are currently no prospectively-validated severity scores for CDI. Recurrence was defined as a new episode of diarrhoea and positive toxin assay within 8 wk after a first correctly-treated episode.

According to French guidelines, a nosocomial CDI was assumed if diarrhoea onset took place more than 2 d after admission to hospital or if hospital admission occurred within 4 wk of discharge and indeterminate or unknown if the patient had been discharged from a healthcare facility within the previous 4-12 wk. Cases were defined as community-acquired if CDI signs presented in the absence of previous hospitalisation within the last 12 wk in out- or in-patients within the first 48 h of admission[33]. French health authorities currently adopt a cut-off period of 48 h post-admission to define hospital-acquired infections. We considered fever as core temperature > 38 ℃, and leukocytosis as leukocyte count > 15 × 10^9/L. Malnutrition was defined according to the national recommendations[34]. The incidence rate was calculated as the number of CDI in MICU per 1000 admitted patients.

Statistical analysis

The data were analysed in 2 stages. First, univariate analysis identified significant differences between severe and non-severe CDI cases. Continuous variables were compared by the Mann-Whitney *U* test. The χ^2 or Fisher's exact test compared categorical variables. Second, a multivariate logistic regression model identified factors associated with CDI severity. The distribution of continuous variables was checked. All potential risk factors significant at the 0.2 level in univariate analysis were entered into the model. Multivariate analysis was performed with models that were judged a priori to be clinically sound. This was prospectively determined to be necessary to avoid producing spuriously significant results with multiple comparisons. The goodness-of-fit was assessed by the Hosmer-Lemeshow test. For all tests performed, 2-tailed *P* values < 0.05 were regarded as denoting statistical significance. Statistical data were analysed with statistical package for the social sciences (version 17.0 for Windows, SPSS, Inc., Chicago, IL).

RESULTS

A total of 40 adult patients suffering from CDI-related diarrhoea diagnosed in MICU from January 2007 and December 2011 were included. The mean incidence rate was 12.94 cases/1000 admitted patients, and 14.93, 8.52, 13.24, 19.70, and 8.31 respectively per 1000 admitted patients annually from 2007 to 2011 (P = 0.99). The demographics and outcomes of these patients are summarised in Table 1. Median age was 62.9 [interquartile range (IQR) 55.4-72.40] years; 13 (32.5%) were women, and 24 (60%) were admitted directly to MICU. Median length of MICU stay was 14.0 d (IQR 5.0-22.8). Twenty-nine patients (72.5%) presented symptoms after MICU admission, and 15 patients (37.5%) developed CDI 10 d or less after MICU admission. Based on the inclusion criteria, ELISA was positive in 35 patients (87.5%), with the remaining 5 patients (12.5%) being diagnosed by toxigenic culture. Median time between onset of symptoms and microbiological diagnosis was 2 d for ELISA and 10 d for toxigenic culture. The mean interval between onset of symptoms and *C. difficile* laboratory test results was 7.2 ± 16.5 d. The duration of diarrhoea was 13.0 (8.0-19.5) d. In addition to diarrhoea, the clinical symptoms of CDI were fever (> 38 ℃) in 23 patients, abdominal pain in 15 patients, and ileus in 1 patient. At the time of diagnosis, median leukocyte count was 14.4 (9.45-21.73), with leukocytosis (> 20 × 10^9/L) in 12 patients (30%). C-reactive protein was 117 mg/L (60-193), and albumin was 26.0 g/L (20.0-28.0). Prior to CDI, 38 patients (95.0%) were exposed to antibiotics, and 12 (30%) received at least 4 antibiotics. Fluoroquinolones, 3rd generation cephalosporins, coamoxiclav and tazocillin were prescribed most frequently (65%, 55%, 40% and 37.5%, respectively). During MICU stay, 12 patients received parenteral nutrition due to malnutrition and impossible intake. The majority of patients had hospital-acquired CDI (90%), with 5 cases (13.9%) being MICU-acquired. Metronidazole was administered as a single agent to 25 patients and vancomycin to 2 (5%). Eight patients (20%) received a combination of 2 CDI medications during the course of treatment. Five patients were given no antimicrobials against CDI.

Of our 40 patients, 15 (37.5%) had severe CDI. Table 1 shows characteristics of severe and non-severe patients. Univariate analysis showed that Glasgow coma score, gender, diabetes mellitus, previous exposure to fluoroquinolones, PPI or coamoxiclav, and C-reactive protein were statistically different between severe and non-severe patients. Multivariate analysis indicated that male gender, C-reactive protein levels, and fluoroquinolones were independently associated with severe CDI (Table 2).

The prognosis of CDI was good in 18 patients. A total of 12 patients (30%) experienced complications due to their infection with 2 cases (16.7%) of pseudomembranous colitis (PMC) and 4 cases (33.3%) of colitis. In one patient, CDI was marked by hyper-leukocytosis (53

Table 1 Comparison of the characteristics of severe and non-severe *Clostridium difficile* infection patients hospitalised in medical intensive care unit between January 2007 and December 2011

	Total n = 40	Severe CDI n = 15	Non-severe CDI n = 25	*P* value
Age (yr)	62.9 (55.3-72.4)	59.52 (54.8-77.3)	64.27 (56.1-72.2)	0.99
Male gender	27 (67.5)	13 (86.7)	14 (56.0)	0.045
Origin of patient				0.61
Home	14 (35.0)	6 (40)	8 (32)	
Other ward and/or other hospital	26 (65.0)	9 (60)	17 (68)	
Diagnosis at MICU admission				0.39
Respiratory disease	15 (37.5)	3 (20)	12 (48)	
Septic shock	12 (30.0)	6 (40)	6 (24)	
Renal disease	3 (7.5)	1 (6.7)	2 (8)	
Gastrointestinal disease	3 (7.5)	1 (6.7)	2 (8)	
Neurological disease	3 (7.5)	1 (6.7)	2 (8)	
Other	4 (10.0)	3 (20)	1 (4)	
Clinical symptoms and biological features at diagnosis				
Fever	23 (57.5)	8 (53.3)	15 (60.0)	0.75
Abdominal pain	15 (37.5)	7 (46.7)	8 (32.0)	0.35
Duration of diarrhoea (d)	13.0 (8.0-19.5)	18 (5-29)	13 (8-17)	0.38
C-reactive protein (mg/L)	117 (60-193)	185 (73-339)	105 (39-127)	0.01
Albumin count (g/L)	26.0 (20.0-28.0)	23 (17-27)	26 (21-28)	0.30
Leukocyte count (x 10^9/L)	14.4 (9.5-21.7)	17.9 (10.6-33.4)	12.4 (9.0-21.1)	0.17
Previous exposure to CDI risk factors within 8 wk before onset of symptoms				
Hospitalisation	28 (70.0)	10 (66.7)	18 (72.0)	0.72
Exposure to PPI	21 (52.5)	10 (66.7)	11 (44.0)	0.17
Chemotherapy	12 (30)	5 (33.3)	7 (28.0)	0.72
Gastrointestinal procedures	23 (57.5)	9 (60.0)	14 (56.0)	0.80
Antibiotic treatment	38 (95.0)	15 (100)	23 (92)	0.26
Cephalosporins 3rd generation	22 (55)	8 (53.3)	14 (56)	0.87
Clindamycin	2 (5)	1 (6.7)	1 (4)	0.71
Coamoxiclav	16 (40)	8 (53.3)	8 (32)	0.18
Fluoroquinolones	26 (65)	13 (86.7)	13 (52)	0.026
Treatment				0.06
No treatment	5 (12.5)	2 (13.3)	3 (12.0)	
Only metronidazole	25 (62.5)	6 (40)	19 (76)	
Only vancomycin	2 (5)	2 (13.3)	0 (0)	
Metronidazole+vancomycin	8 (20)	5 (33.3)	3 (12.0)	
Duration of hospital stay (d) and outcomes				
LOS in hospital	27.0 (13.5-50.8)	16 (5-48)	28.0 (16.0-55.5)	0.26
LOS in MICU	14.0 (5.0-22.8)	8 (2-21)	16.0 (6.0-25.5)	0.27
Death in 30 d	16 (40)	9 (60)	7 (28)	0.046

Data represent *n* (%) of patients for categorical variables and median (interquartile range) for continuous variables. CDI: *Clostridium difficile* infection; LOS: Length of stay; MICU: Medical intensive care unit; PPI: Proton pump inhibitor; WBC: White blood cells.

g/L), PMC, renal failure and intestinal perforation. The patient died 56 d after CDI diagnosis.

Overall mortality was 52.5%; 12 patients expired in MICU and 9 in-hospital after MICU discharge. The mortality rate within 30 d after diagnosis was 40%; 9 deaths (56.3%) were CDI-related according to the physician in charge of the patient.

DISCUSSION

C. difficile acquisition and severe CDI development are primarily associated with healthcare, although severe, community-acquired infections among persons previously thought to be at low risk have been reported[35,36]. CDI management has become more daunting over the past decade because of alarming increments in CDI incidence and severity. These increases have caused significant, concomitant escalation of the healthcare economic burden from CDI and will likely translate into excessive ICU admissions and attributable mortality. Up to 20% of critically-ill patients may suffer from ileus without the diarrhoea typically associated with CDI[37]. The absence of diarrhoea coupled with the inability of critically-ill patients to communicate with care providers make the diagnosis of CDI extremely difficult[38]. The objectives of this study were to analyse factors associated with CDI severity and to describe the prognosis of CDI in hospitalised MICU patients.

Our investigation comprised 40 CDI patients diagnosed at a MICU between 2007 and 2011, with a mean incidence rate of 12.94 cases/1000 admitted patients. All included cases had their first episode of CDI. The majority were hospital-acquired (90%), with 5 cases (13.9%) being MICU-acquired. In this work, we compared the characteristics of a group of 15 cases of severe CDI with a group of 25 patients without severe CDI in our MICU.

Table 2 Factors independently associated with severe *Clostridium difficile* infection among patients in medical intensive care unit

Variables	Unadjusted OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
Glasgow coma score	1.16 (0.99-1.36)	0.15	-	
Diabetes mellitus	4.89 (1.00-23.93)	0.04	-	
Previous PPI exposure	2.55 (0.67-9.66)	0.17	-	
Coamoxiclav (in the previous 8 wk)	2.43 (0.65-9.07)	0.18	-	
Fluoroquinolones (in the previous 8 wk)	6.0 (1.12-32.28)	0.026	9.29 (1.16-74.28)	0.036
C-reactive protein (mg/L; 10 mg/L increments)	1.10 (1.02-1.18)	0.014	1.11 (1.02-1.21)	0.021
Male gender	5.11 (0.95-27.55)	0.045	8.45 (1.06-67.16)	0.044

Exposure to fluoroqionolones, C-reactive protein level and gender were included in the multivariate model [The value of the likelihood was 34.56 with 3 df, and χ^2 test: 18.37 (P < 0.0001)]. OR: Odds ratios; PPI: Proton pump inhibitor.

In univariate analysis, gender, BMI, diabetes mellitus, fluoroquinolone use and C-reactive protein were associated with CDI severity. Multivariate logistic regression modelling showed that male gender, C-reactive protein, and previous exposure to fluoroquinolones were independently linked with severe CDI. Exposure to specific antimicrobial drugs, notably fluoroquinolones, clindamycin, and cephalosporins, has been linked to severe CDI in some studies[3,21] but not in others[14].

Malnutrition, reported to be as high as 40%, is prevalent in ICU patients and is associated with increased morbidity and mortality[39], but to the best of our knowledge, this observation has not been made in CDI patients. The majority of patients were not referred to a dietitian. Among patients consulting a dietitian, 87.5% required parenteral nutrition, which was not associated with 1-month survival in our study. This is consistent with the findings of a previous meta-analysis of 26 randomised trials[40]. The investigators showed that, in critically-ill patients, parenteral nutrition did not influence overall mortality.

Underlying illness is moderately associated with severe CDI[14], an effect not observed in our study and could be related to the homogeneity of our study population. Recent investigations have disclosed a potential role of acid suppression in CDI acquisition and relapse[41,42]. Hardt *et al*[43] noted an association between these agents and severe CDI, although their definition of severe CDI was different. Also, significant linkage has been reported in a recently-published paper[44]. This effect was not seen in our study, but may be related to our study population, and PPIs did not play a role in CDI severity in MICU. Previous works have identified few clinical characteristics that consistently predict severe CDI. Different findings, such as fever, abdominal pain, decreased albumin, and significant leukocytosis (often > 20 g/L), are likely in severe colitis[45,46]. Such outcomes often precede multi-organ dysfunction and should prompt urgent consideration of CDI as a possible cause[47,48]. In our study, these variables were not different between severe and non-severe cases. Ananthakrishnan *et al*[49] demonstrated that serum albumin < 3 g/dL, haemoglobin < 9 g/dL and creatinine > 1.5 g/dL were independent predictors of severe CDI and may have prognostic significance in patients with inflammatory bowel disease. We also identified rising serum C-reactive protein levels as being independently associated with severe CDI. As the distribution of C-reactive protein was normal, our multivariate result suggested that an increase by 10 mg/L lead to an increase of the risk of severe CDI by 10%. In fact, serum C-reactive protein was a far better predictor of severe CDI than white blood cell count, which has been implicated by others[43,50-52]. Perhaps more sensitive markers of inflammation, such as procalcitonin, might be especially useful in the evaluation of disease severity. Male gender was associated with severe CDI; to the best of our knowledge, this has not been found in other series. However, a similar effect was reported in a Canadian study, where women were less likely to develop severe CDI, but it was indicated by univariate analysis and was not significant[53]. Our study provides data on the initial treatment courses chosen by care providers. The majority of patients were treated with metronidazole. Only 7 (46.7%) with severe disease received vancomycin. However, information regarding antibiotherapy (duration and dosage) of CDI was not fully captured; thus, the treatment response could not be analysed in our study. Although current guidelines from the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America urge vancomycin as first-line therapy in severe disease among adult patients[54], a major portion of our study period predated the publication of these recommendations. In contrast, severe CDI was not associated with nursing home residency, the presence of hospital-acquired CDI or increasing age.

Our study has some limitations which need to be considered when interpreting the data. Our sample size was limited and the study was conducted in one single hospital. Therefore we would not be able to extrapolate our results to other groups. Other potential predictors of severe CDI were unable to provide complete risk scores. There was no validated definition of severe CDI; thus, we applied criteria of severe CDI without a scoring system. A larger, multi-center study would be required to validate any definition of severe CDI. Our patients were assembled from a MICU in a tertiary hospital and may not be generalisable to patients in community hospitals or outpatient settings. Our study population consisted of a significant proportion of patients with multiple co-morbidities, which may reflect tertiary care settings. However, these

centers may be ideal to investigate severe CDI, as patients at risk of severe disease are usually found in tertiary care facilities. The number of antibiotic days should be considered as a potential risk factor for severity, which was not available in our data. Nevertheless, we could not detail the antibiotic consumption. Instead we simply noted if antibiotics were used in the last 2 mo preceding CDI.

We performed this study with the aim of identifying factors that predict severe outcomes associated with CDI in MICU patients. Our results indicate that low C-reactive protein, male gender and previous use of fluoroquinolones are independent predictors of severe CDI in hospitalised MICU patients. In the majority of published studies, factors for a severity score index of CDI were assessed within 48 h after laboratory reporting of test results positive for *C. difficile*. This is problematic in terms of reproducibility in deciding the severity score index of CDI, because the time window from CDI diagnosis to the evaluation of severe CDI is variable. We reported predictors of severe CDI not dependent on the timing of their assessment except for C-reactive protein; in our study, however, values were obtained from the day of CDI diagnosis which made these results valid in clinical practice.

Identification of such factors would foster the development of a quantitative severity score that could drive comparative effectiveness investigations and prospective trials of CDI therapy in these patients. Clinicians need to maintain a high index of suspicion and must often rely on physical examinations and laboratory findings to make the diagnosis. Vancomycin is recognized as the first-line treatment of severe CDI and should be preferred in the ICU setting. Rigorous attention to infection control measures and vigorous antimicrobial stewardship are essential to prevent *C. difficile* transmission. Improved diagnostic methods and new therapeutic tools are required to help clinicians to manage severe CDI cases.

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COMMENTS

Background

Clostridium difficile infection (CDI) has become a growing cause of nosocomial morbidity, high hospital costs and mortality over the world. Several mechanisms have been postulated to increase disease severity, including the emergence of hypervirulent strains. Critically-ill patients are at particularly high risk of CDI due to the prevalence of multiple risk factors in the patient population. However, factors associated with CDI severity in medical intensive care unit (MICU) are poorly documented.

Research frontiers

Current data are dealing with many aspects related to CDI. The list of hotspots, not exhaustive in any standards, would include measures of prevention, modalities of diagnosis and treatment, and standardization of basic definitions including severity and evaluation scales. Defining a set of approved prognostic factors would help us dealing with aforementioned topics.

Innovations and breakthroughs

The authors reported predictors of severe CDI no matter the timing of assessment except for C-reactive protein. Nevertheless, values were obtained from the day of CDI diagnosis which made these results valid in clinical practice.

Applications

Identification of some factors would foster the development of a quantitative severity score that could drive comparative effectiveness investigations and prospective trials of CDI therapy in patients hospitalised in MICU. Intensivists need to maintain a high index of suspicion and must often rely on physical examinations and laboratory findings to make the diagnosis.

Peer review

Risk factor assessment limited due to small sample size, but a tremendous time investment into the statistical analysis of this small sample makes the manuscript interesting. The study design is simple and reasonable and statistics are excellent.

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BRIEF ARTICLE

Prognosis and follow-up of 135 patients with ischemic colitis over a five-year period

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Abstract

AIM: To study the prognosis (recurrence and mortality) of patients with ischemic colitis (IC).

METHODS: This study was conducted in four Spanish hospitals, participants in the Ischemic Colitis in Spain study We analyzed prospectively 135 consecutive patients who met criteria for definitive or probable IC according to Brandt criteria, and follow up these patients during the next five years, retrospectively. Long-term results (recurrence and mortality) were evaluated retrospectively after a median interval of 62 mo (range 54-75 mo).

RESULTS: Estimated IC recurrence rates were 2.9%, 5.1%, 8.1% and 9.7% at years 1, 2, 3 and 5 years, respectively. Five-year survival was 69% (93 of 135) and 24% (10 of 42 patients) died for causes related to the IC. Among these 10 patients, 8 died in their first episode at hospital (4 had gangrenous colitis and 4 fulminant colitis) and 2 due to recurrence.

CONCLUSION: The five-year recurrence rate of IC was low. On the other hand, mortality during follow-up was high and was not associated with ischemic colitis.

Key words: Colonic; Ischemic; Recurrence; Follow-up; Mortality

Core tip: The prognosis of patients with ischemic colitis is unknown. In this study we observed that recurrence rate of ischemic colitis was low (9.7% at 5 years). However, the mortality was high (31% at 5 years) and the only factor associated with mortality was age.

Cosme A, Montoro M, Santolaria S, Sanchez-Puertolas AB, Ponce M, Durán M, Cabriada JL, Borda N, Sarasqueta C, Bujanda L. Prognosis and follow-up of 135 patients with ischemic colitis over a five-year period. *World J Gastroenterol* 2013; 19(44): 8042-8046 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8042.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8042

INTRODUCTION

Ischemic colitis (IC) has been estimated to account for approximately 3 in 1000 of all admissions to tertiary hospitals[1]. In a recent prospective study conducted in 24 spanish hospitals, IC was the reason for 1.28 per 1000 hospital admissions[2]. According to the literature, the incidence in the general population is of 4.5-9.9 cases per 10^5 people/year and of 44 per 10^5 people/year for those above 40 years of age[3]. Many cases of IC (reversible forms) are ignored and undiagnosed.

The number of comorbid disorders ($\geqslant$ 5)[4], location in the right colon[5], and certain clinical onset (gangrenous colitis and/or fulminant pancolitis)[2] are known to be associated with poor prognosis of the disease. However, only a few number of studies, have analysed the long-term prognosis of these patients. The objective of this study was to assess the prognosis (recurrence and mortality) of our patients with IC after first hospital admission.

MATERIALS AND METHODS

We assessed the long-term recurrence and mortality of patients in four hospitals participating in the Ischemic Colitis in Spain (CIE) study (San Jorge Hospital in Huesca, Donostia Hospital in San Sebastian, La Fe Hospital in Valencia and Galdakao Hospital in Bizkaia). The CIE study is a prospective multicentre study which consecutively included all patients with diagnosis of IC between March 2005 and December 2006[2].

For our study, patients follow-up was continued until June 2011. Data were obtained retrospectively from the outpatient clinic (gastroenterology and/or surgery) or by telephone using a questionnaire given to the patient and/or their families. The mean follow-up period was 62 mo (54-75 mo).

Patients were categorized as having definitive, probable, or possible IC according to the Brandt criteria[6-8]. The clinical pattern and outcome for each patient was categorised according to Brandt and Boley classification[6]: (1) reversible colopathy; (2) transient colitis; (3) chronic segmental IC; (4) gangrenous colitis; or (5) fulminant universal colitis.

The Ethics Committee of the Clinic Hospital of Barcelona approved the study protocol on 9 June 2005. All patients gave their written consent. The initial study protocol included, in all cases, a colonoscopy before the patient was released from the hospital. A second colonoscopy was not performed in asymptomatic patients and those with reversible colopathy or transient colitis, as these subclasses of disease heal spontaneously.

Statistical analysis

To compare differences between the groups, the Fisher test was used for qualitative variables and the Student's *t*-test for quantitative variables. Differences were considered to be significant when P values were below 0.05.

RESULTS

A total of 135 patients with who met IC criteria were included in the study. The diagnosis of IC was definitive in 74% and probable in 26% of these cases. The average age of our cohort was 73 ± 10 years with a range of 17-90 years, and 50% of them were women. Of the 135 patients included in the study, 51 (38%) were classified with reversible colopathy; 29 (21%) transient colitis; 42 (31%) chronic segmental IC; 9 (7%) gangrenous colitis, and 4 (3%) fulminant universal colitis.

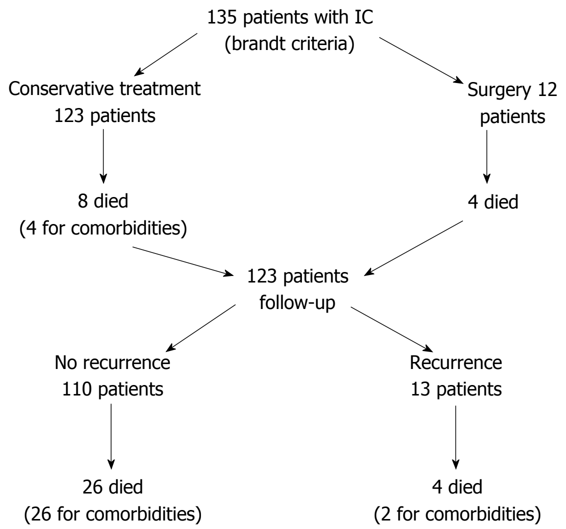
A total of 16 (12%) of the 135 patients had an unfavourable outcome namely death and/or the need for surgery. In the first episode at hospital, 12 patients (8.8%) died, 8 due to IC and 4 due to comorbidities after the acute episode had been solved (1-2 mo after) (Figure 1). Among the 8 who died from IC (3 fulminant universal colitis and 1 gangrenous colitis operated with a subtotal colectomy, and 1 fulminant universal colitis and 3 gangrenous colitis non-operated) the outcome reflected the severity of disease: gangrenous colitis (4/9, 44.4%) and fulminant pancolitis (4/4, 100%).

IC recurred during the follow-up period in 13 (9.7%) of the 123 patients (Tables 1 and 2). Patients with IC recurrence had a similar clinical onset at the first episode at hospital and at relapse. All these patients had colitis not gangrenous and had more frequently vomiting and abdominal pain in the first episode. These were non-grangrenous forms of colitis and located mainly in the left colon. Recurrence occurred in 4, 3, 4, 2 cases through the first, second, third and five year of follow-up, respectively.

Considering the course of the 123 patients, 30 (24.3%) of them died within 5 years, 28 due to comorbidities and only 2 due to recurrence and complications of the IC. The only factor that was associated increased mortality was age (72.5 ± 11 years *vs* 78.6 years; $P < 0.001$). There were no differences by sex, nonsteroidal anti-inflammatory drugs (NSAIDs) intake or recurrence of colitis. Patients who died had more frequently hypertension 38.8% *vs* 22.4% ($P = 0.06$). Among the 2 patients who died of recurrent IC, one, had reversible colitis, two months after stent insertion for chronic mesenteric ischemia, and the other had chronic segmental colitis, after 24 mo of mesenteric angina. The overall five-year survival was 69% (93 of 135 patients).

Chronic segmental ischaemic colitis

The patients with chronic segmental IC were followed-up for six months with periodic colonoscopies and/or barium enemas. Of the 42 patients, 30 remained asymptomatic; 6 (14%) developed a stenosis; 3 (7%) had continuing or recurrent bloody diarrhoea and 1 (2%) suffered from persistent or chronic diarrhoea with protein-losing colopathy and serum albumin levels < 2.8 g/L. After six



Study of 364 patients with ischemic colitis in Spain

Figure 1 Outcome of 135 patients with ischemic colitis after initial treatment. IC: Ischemic colitis.

Table 1 Clinical features and clinical pattern of the ischemic colitis *n* (%)

Symptoms	No recurrence[1] (*n* = 110)	Recurrence[2] (*n* = 13)	*P* value
Acute abdominal pain	76 (69)	12 (92)	< 0.05
Haematochezia	90 (82)	11 (85)	0.4
Diarrhoea	41 (37)	6 (46)	0.2
AUBD sequence	57 (52)	7 (54)	0.4
Vomiting	20 (18)	5 (38)	< 0.05

[1]Fulminant colitis 4, gangrenous colitis 8 and non-gangrenous colitis 98 (transient colitis 22, reversible colitis 42 and chronic segmental colitis 34); [2]Transient colitis 5, reversible colitis 4 and 4 chronic segmental colitis. AUBD: Sequence of abdominal pain, urgent desire to defecate, and bloody diarrhoea.

months none of these patients reported complications.

DISCUSSION

IC is the most common form of intestinal ischemia[9]. It is predominantly observed in elderly patients with varying comorbidities, though younger individuals may also be affected. The mean incidence of autopsy-verified fatal IC has been estimated to be $1.7/10^5$ person years, rising to $23/10^5$ person years in octogenarians[10]. In our study, the average age was 73 years, and clinical onset of IC was observed equally frequent in both women and men.

Clinical presentation in ischemic colitis varies, depending of the severity and extent of the disease. In general, the first symptoms of IC are haematochezia and acute abdominal pain[2,11-14]. Any part of the colon may be affected in IC although the left colon is the predominant location in approximately 75%-85% of patients[12-14]. Splenic flexure is involved in nearly one-quarter of patients[15-17], and isolated right colon ischemia (IRCI) in about 10%-26% of cases[6,17]. Right-sided colonic ischemia tends to be more severe: about 60% of patients require surgery (four or five times more than with colitis in other areas). In our cohort, of the 10 patients with the right colon involved, 6 (5 with gangrenous colitis) required surgery.

Table 2 Univariate analysis of variables in patients with ischemic colitis without recurrence versus patients with recurrence *n* (%)

	No recurrence (*n* = 110)	Recurrence (*n* = 13)	*P* value
Men/women	56/54	6/7	0.8
Age ≥ 65 yr	91 (83)	12 (92)	0.7
Age ≥ 80 yr	32 (29)	4 (31)	1
Hypertension	66 (60)	10 (77)	0.4
Patients under NSAID treatments	32 (29)	5 (38)	0.5
≥ 3 comorbidities diseases[1]	43 (39)	4 (31)	0.8
Location			
Pancolitis	4 (3)	0 (0)	1
≥ 2 locations	45 (41)	5 (38)	1
Caecum	6 (5)	1 (8)	0.5
Ascending colon	7 (6)	1 (8)	0.6
Hepatic flexure	8 (7)	1 (8)	1
Transverse colon	8 (7)	1 (8)	1
Splenic colon	23 (21)	3 (23)	1
Descending colon	45 (41)	2 (15)	0.08
Sigmoid colon	74 (67)	11 (85)	0.0001
Rectum	17 (15)	1 (8)	0.7
Clinical presentations			
Non-gangrenous	98 (89)	13 (100)	0.8
Gangrenous	8 (7)	0 (0)	0.6
Fulminant	4 (3)	0 (0)	1

[1]Comorbidities (diabetes, 44 patients; dyslipidaemia, 36; ischaemic heart disease, 34; cerebrovascular disease, 29; atrial fibrillation, 28; peripheral vascular disease, 24; congestive heart failure, 11; recent arterial hypertension, 11; malignancy, 3 and miscellaneous, 15). NSAID: Nonsteroidal anti-inflammatory drug.

It has been estimated that about 20% of patients with acute IC will require surgery with an associated mortality rate of up to 60%[18-21]. In our study, 12 (9%) of the 135 patients underwent surgery, 3 with universal fulminant colitis, 6 with gangrenous colitis, 2 with chronic segmental colitis and 1 with transient colitis. The gangrenous and fulminant universal colitis are associated with poorer prognosis than non-gangrenous forms of IC. Global mortality of IC is of 8%-10%. Gangrenous forms mortality usually reaches 30% and universal colitis is near 100%.

The rate of the recurrence of IC has been reported to be 10%-16% within five years[11,22]. To assess the recurrence, we evaluated the long-term outcomes in our patients (54-75 mo). Thirteen patients had recurrent symptoms. Two of them, one presented chronic segmental colitis and another with transient colitis did not have sigmoid colon involved. There were no statistically significant differences between the clinical presentation of IC and the involvement of different segments of the left colon. The estimated cumulative recurrence rates at years, 1, 2, 3 and 4/5 were 2.9%, 5.1%, 8.1% and 9.7%, respectively.

Some authors[23,24] have recommended that the following studies should be carried out on a prospective basis to assess potential etiologic factors that may increase the likelihood of recurrence: hypercoagulability workup, tests for connective tissue disorders, echocardiogram and holter, and magnetic resonance angiography. In this way, we may be able to detect structural heart diseases, fibromuscular dysplasia and other conditions that may predispose individuals for IC or to recurrence.

The question of whether patients should receive prophylactic treatment for recurrent IC after discharge from the hospital is important. Currently, our efforts should be addressed to control those factors that may contribute to develop IC such as intake of NSAID[25,26] and vasoactive drugs and arterial hypertension.

The limitations of this study are those of a retrospective analysis but prospective follow-up patients. The data collection was made by telephone or at the outpatient clinic and colonoscopy was undertaken only if symptoms were consistent with IC.

In summary, IC is associated with age and occurrence on the right-side markedly increases the risk of severe disease that requires surgery or leads to death. The mortality rate of IC is still high and the recurrence increases with time. In our sample, mortality due to IC at the first admission was 5.9% and 7.4% five years later. The overall rates of mortality, including comorbidities were 8.8% and 31.1% respectively.

ACKNOWLEDGMENTS

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COMMENTS

Backgrounds

Ischemic colitis (IC) is the most common form of intestinal ischemia. IC is predominantly observed in elderly patients with varying comorbidities, although younger individuals may also be affected. IC was the reason for 1.28 per 1000 hospital admissions.

Research frontiers

Few studies have assessed the long-term prognosis of these patients.

Innovations and breakthroughs

IC is associated with age and occurrence on the right-side markedly increases the risk of severe disease that requires surgery or leads to death. The mortality rate of IC is still high and the recurrence increases with time. In our study, mortality due to IC at the first admission was 5.9% and 7.4% five years later. The overall rates of mortality, including comorbidities were 8.8% and 31.1% respectively.

Applications

Patients with ischemic colitis should be monitored continuously to prevent decompensation during follow-up.

Peer review

It is a clinical series of IC. It is well written.

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BRIEF ARTICLE

Single balloon enteroscopy for endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejuno anastomosis

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Abstract

AIM: To evaluate single balloon enteroscopy in diagnostic and therapeutic endoscopic retrograde cholangiography (ERC) in patients with Roux-en-Y hepaticojejunoanastomosis (HJA).

METHODS: The study took place from January 2009 to December 2011 and we retrospectively assessed 15 patients with Roux-en-Y HJA who had signs of biliary obstruction. In total, 23 ERC procedures were performed in these patients and a single balloon videoenteroscope (Olympus SIF Q 180) was used in all of the cases. A transparent overtube was drawn over the videoenteroscope and it freely moved on the working part of the enteroscope. Its distal end was equipped with a silicone balloon that was inflated by air from an external pump at a pressure of ≤ 5.4 kPa. The technical limitations or rather the parameters of the single balloon enteroscope (working length - 200 cm, diameter of the working channel - 2.8 mm, absence of Albarran bridge) showed the need for special endoscopic instrumentation.

RESULTS: Cannulation success was reached in diagnostic ERC in 12 of 15 patients. ERC findings were normal in 1 of 12 patients. ERC in the remaining 11 patients showed some pathological changes. One of these (cystic bile duct dilation) was subsequently resolved surgically. Endoscopic treatment was initialized in the remaining 10 patients (5 with HJA stenosis, 2 with choledocholithiasis, and 3 with both). This treatment was successful in 9 of 10 patients. The endoscopic therapeutic procedures included: balloon dilatation of HJA stenosis - 11 times (7 patients); choledocholitiasis extraction - five times (5 patients); biliary plastic stent placement - six times (4 patients); and removal of biliary stents placed by us - six times (4 patients). The mean time of performing a single ERC was 72 min. The longest procedure took 110 min and the shortest took 34 min. This shows that it is necessary to allow for more time in individual procedures. Furthermore, these procedures require the presence of an anesthesiologist. We did not observe any complications in these 15 patients.

CONCLUSION: This method is more demanding than standard endoscopic retrograde cholangiopancreatography due to altered postsurgical anatomy. However, it is effective, safe, and widens the possibilities of resolving biliary pathology.

Key words: Single balloon enteroscopy; Endoscopic retrograde cholangiography; Roux-Y hepaticojejunoanastomosis; Endoscopic diagnosis; Endoscopic treatment

Core tip: Endoscopic retrograde cholangiopancreatography (ERCP) represents a demanding method even in a normal anatomical situation. When a surgically altered gastrointestinal or pancreatobiliary anatomy is present, ERCP becomes even more demanding. Our retrospective study assessed diagnostic and therapeutic endoscopic retrograde cholangiography (ERC) using a single balloon enteroscope in 15 patients with Roux-en-Y hepaticojejunoanastomosis. A comparatively high success rate was achieved in both diagnostic (80%) and therapeutic (90%) ERC. This method is both time-consuming and technically demanding. However, it is an effective and safe method that widens the possibilities of resolving biliary pathology in these conditions.

Kianička B, Lata J, Novotný I, Dítě P, Vaníček J. Single balloon enteroscopy for endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejunoanastomosis. *World J Gastroenterol* 2013; 19(44): 8047-8055 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8047.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8047

INTRODUCTION

Hepaticojejunoanastomosis (HJA) construction is a frequent method of surgical bypass for resolution of pathological conditions of the extrahepatic bile ducts, which enables bile drainage into the small intestine. These are the main groups of indications for HJA construction: (1) benign pathological processes in the papilla of Vater (VP) and terminal common bile duct, which cannot be resolved by endoscopy or surgical resection; (2) local inoperable pathological processes in the VP and distal part of the hepatocholedoch that cannot be resolved radically in patients who are able to undergo surgery; (3) stenoses of the distal part of the hepatocholedoch during expansion of the pancreas (both malignant and benign) stenosing the common bile duct; (4) iatrogenic or traumatic damage of the hepatocholedoch (*e.g.*, after laparoscopic cholecystectomy) leading to bile leak or stenosis that cannot be resolved by endoscopic retrograde cholangiopancreaticography (ERCP) or percutaneous transhepatic cholangiography (PTC), leaving a proximal section of the hepatic duct long enough to enable anastomosis; (5) HJA stenosis; and (6) congenital bile duct anomaly with proximal section of the extrahepatic bile ducts in good condition.

Bile duct injuries during laparoscopic and open cholecystectomy belong to the most serious iatrogenic injuries, with high morbidity and mortality. The increasing number of laparoscopic cholecystectomies has led to an increase in the number of bile duct injuries. Early perioperative detection of these injuries serves as the basis of successful reconstruction of the bile duct where HJA on Roux loop is the gold standard[1]. This procedure is nowadays a standard way of treating injuries of the bile duct and its consequences in the form of stenoses. HJA performed using Roux-en-Y loop (less frequently using omega loop) can be used universally. In general, using this method of bile duct reconstruction for any indication does not lead to mistakes. In contrast to simple end-to-end anastomosis, HJA can be used at any time, in the case of loss-making injury of the bile duct (*e.g.*, excision of its part) or in the case of thin and fine bile duct. HJA can be used in all recent injuries to the bile duct as well as in all adjustments of its stenoses (*i.e.*, in chronic conditions)[2]. It is controversial to perform reoperations for complications of laparoscopic procedures of the bile duct. For reoperations, it is possible to use the da Vinci robotic system, which offers a wide range of visualizing possibilities (fluoroscopy) and movability of instruments (endowrist)[3-5]. The main condition necessary for long-term successful diagnostic results and results of treatment of hepatobiliary diseases is the multidisciplinary approach of surgeons, endoscopists, and interventional radiologists.

One of the serious postoperative complications of HJA is stenosis, with the possible development of cholangitis. Surgery is frequently performed in the unfavorable conditions of inflammatory changes. It is therefore clear that the percentage of restenosis in the area of HJA is comparatively high and reaches about 7% even in the best institutions[6].

The HJA construction (mainly on Roux-en-Y or less frequently on the omega intestinal loop) causes the bile duct orifice into the small intestine to become unreachable by ERCP performed in the standard way (*i.e.*, by lateroscope). That is why biliary drainage used to be provided using the transhepatic approach (*i.e.*, PTC) or surgically.

These patients with Roux-en-Y HJA and with signs of biliary obstruction have therefore in the past been a challenge for endoscopists due to the absence of endoscopic access to enterobiliary anastomosis[7,8].

Single balloon enteroscopy has proved to be effective for deep intubation of the small intestine. The basic technique of performing single balloon enteroscopy has been described extensively in the literature[9,10].

The use of a balloon enteroscope (initially a double balloon and recently also a single balloon) has resulted in achievement of enteroenteroanastomosis, and then bilioenteral anastomosis at the distal end of the afferent intestinal loop[11]. ERC was performed in our group of patients with Roux-en-Y HJA by single balloon enteroscopy. Using standard ERCP, in comparison with a lateroscope, one has to take account of certain technical limitations caused by the current balloon enteroscopes: (1) extreme working length of a single balloon enteroscope -

200 cm; (2) small diameter of the working channel of the single balloon enteroscope - 2.8 mm; and (3) absence of Albarran bridge in a single balloon enteroscope. These technical limitations or parameters show the necessity to use suitable endoscopic instrumentation.

ERCP is considered to be a technically demanding procedure of digestive endoscopy and the presence of surgically altered gastrointestinal or pancreatobiliary anatomy makes it even more difficult[12,13].

The aim of this retrospective study was to analyze and evaluate our experience in using single balloon enteroscopy in diagnostic and therapeutic ERC in patients with Roux-en-Y HJA.

MATERIALS AND METHODS

Patients

The study took place from January 2009 to December 2011 and we retrospectively assessed 15 patients (7 men, average age: 55 years; 8 women, average age: 53 years) with Roux-en-Y HJA, who had signs of biliary obstruction.

ERC procedure

Altogether 23 ERC procedures were performed in these 15 patients with Roux-en-Y HJA using a single balloon videoenteroscope (Olympus SIF Q 180). Its working length was 200 cm, the outer diameter was 9.2 mm, and the diameter of the working channel was 2.8 mm. A transparent overtube was drawn over a single balloon enteroscope and it freely moved on the working part of the enteroscope. The overtube was 13.2 mm in diameter and 140 cm long. The distal end was equipped with a silicon balloon that was filled with air from an external pump up to a maximum pressure of 5.4 kPa. Inflation and deflation of this silicon balloon were performed by means of the external pump control.

The examination was performed after a 12-h fast. This endoscopic procedure was both time consuming and technically demanding and required the presence of an anesthesiologist. The mean time to perform the procedure was 72 min. The longest procedure took 110 min and the shortest took 34 min.

During the procedure, the patient lay on the left side and received intravenous sedation (mainly in various combinations) with: midazolam 1-5 mg, sufentanil 5-10 μg, and propofol 20-40 mg repeatedly, to a maximum dose of 200 mg. Buscopan was used after reaching the blind end of the afferent intestinal loop when looking for the HJA orifice.

As can be seen in the schematic image with Roux-en-Y HJA (Figure 1), if an endoscopist wishes to reach the target location, that is, the orifice of the HJA, he/she needs to cover a long distance using a single balloon enteroscope - namely the esophagus, stomach, duodenum, duodenojejunal flexure, proximal jejunum, enteroenteroanastomosis, and afferent intestinal loop, where, at its distal end, 5-6 cm before the blind end of the intestinal loop, lies the orifice of the HJA. Non-ionic iodinated contrast medium (Omnipaque 300) was used for X-ray imaging of the biliary system.

As already mentioned above, the technical limitations or parameters of the single balloon enteroscope (working length - 200 cm, diameter of the working channel - 2.8 mm, absence of Albarran bridge) necessitate the use of special endoscopic instrumentation.

First, a cannula (width 6 Fr and length 330 cm, or width 7 Fr and length 312 cm; Cook Co., Bloomington, IN, United States) was used for cannulation of the orifice of the HJA and the adjoining bile ducts. Later, a triple-lumen extraction balloon was used more frequently for cannulation of the orifice of the HJA, which enabled simultaneous application of the contrast medium and insertion of the guidewire, which made cannulation significantly more effective. This triple-lumen extraction balloon is described below, and it is used, in addition to cannulation of the orifice of the HJA, for endoscopic extraction of choledocholithiasis.

An especially long guidewire of 600 cm (width 0.035 inches; Cook) was used specifically for this type of procedure using a single balloon enteroscope. HJA stenosis was endoscopically dilated by a bougie dilator (7 Fr) and by a balloon dilator (Cook Co., dilatation balloon type QBD, diameter 10 mm, balloon length 3 cm, designated for 2.8 mm diameter working channel, total length of instrument 320 cm). This balloon dilator was used for dilation under a pressure of 3 atm for 2 min.

Choledocholithiasis was endoscopically extracted using the above mentioned triple-lumen extraction balloon (Cook, TXR- HE, width 6.6 Fr, length 275 cm). Plastic biliary drains - width 7 Fr (and in 1 patient also 8.5 Fr), length 3-5 cm (Medinet or Cook or MSA) - were inserted endoscopically. Apart from others, Cook pusher, width 7 Fr, length 320 cm, was used. In cases of biliary obstruction, these inserted biliary drains were endoscopically removed using a polypectomy loop (Olympus).

Ethics

The study was performed in accordance with the ethical criteria of the Declaration of Helsinki. The study was reviewed and approved by the Ethics Committee of St. Anne's University Hospital Brno. Written informed consent to perform diagnostic and therapeutic ERC using a single balloon enteroscope was obtained from all the patients.

RESULTS

In the majority of patients (12/15), HJA construction was required for iatrogenic lesions of the common bile duct after laparoscopic cholecystectomy. In the remaining three patients HJA was required: (1) after resection of the head of the pancreas for chronic pancreatitis; (2) congenital malformation of the common bile duct; and (3) after orthotopic liver transplantation (OLT) for primary sclerosing cholangitis (PSC). Our patients are described

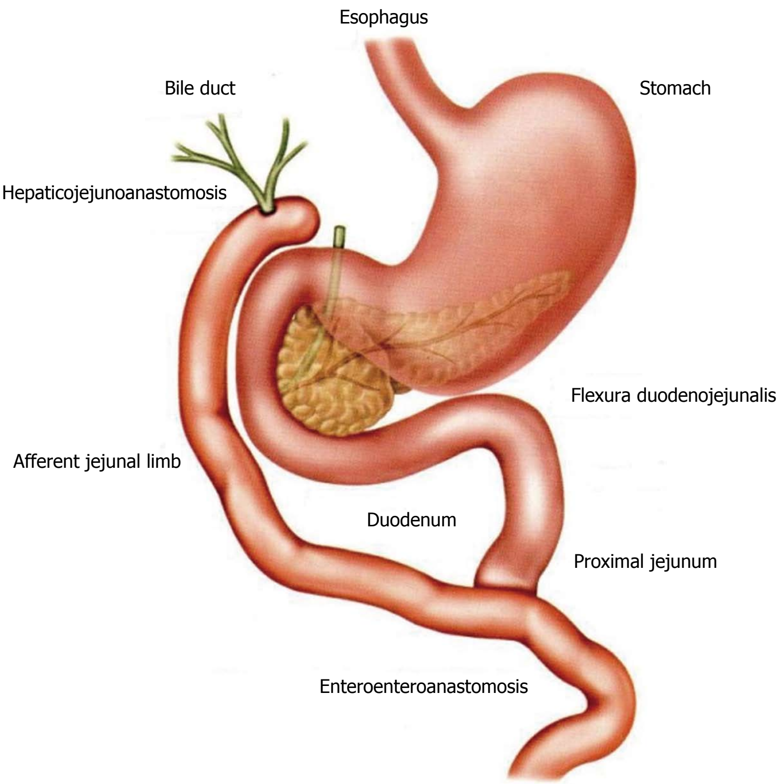

Figure 1 Schematic image of Roux-en-Y hepaticojejunoanastomosis[22].

in Table 1 and a detailed description of each patient follows below.

Patient 1

ERC was performed three times in patient 1 (49-year-old woman). A narrow HJA stenosis was found during the first ERC procedure. Endoscopic balloon dilation of this HJA stenosis was subsequently performed, followed by endoscopic insertion of an 8.5 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. Another ERC procedure was performed under the same conditions 1 mo later. No complications occurred during this month. Initially, an 8.5 Fr biliary drain was endoscopically extracted. After that, a control ERC was performed, showing that the original HJA stenosis was less prominent. Subsequently, repeat endoscopic balloon dilation of the HJA stenosis was performed. At the end of the second ERC session, an 8.5 Fr plastic biliary drain was repeatedly inserted into the bile duct. A third ERC was performed under the same conditions 1 mo later. An 8.5 Fr biliary drain was endoscopically extracted and a control ERC was performed, which showed a smaller HJA stenosis compared with the previous ERC. Another endoscopic balloon dilation of the small HJA stenosis was performed followed by control ERC. The results were satisfactory, showing almost no HJA stenosis. The procedure was then finished. Both the diagnostic and therapeutic ERC in this patient were therefore successful. The patient had no complications after the first ERC.

Patient 2

ERC was performed once in patient 2 (64-year-old woman) and there was only a slight manifestation of HJA stenosis. Endoscopic balloon dilation of this HJA stenosis was subsequently performed followed by control ERC, with satisfactory results and absence of HJA stenosis. Both the diagnostic and therapeutic ERC were therefore successful.

Patient 3

Successful diagnostic ERC was performed once in patient 3 (26-year-old woman) and showed cystic dilation of the bile duct. The condition was primarily resolved surgically, that is, without any attempt to perform endoscopic therapy.

Patient 4

The orifice of the HJA was found in patient 4 (60-year-old man). However, the attempt to probe the orifice was not successful, therefore, ERC was not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and percutaneous transhepatic drainage (PTD).

Patient 5

ERC was performed once in patient 5 (67-year-old man) and it showed slight choledocholithiasis. Endoscopic extraction of the choledocholithiasis using a balloon followed. Both the diagnostic and therapeutic ERC were

Table 1 Detailed description of 15 patients with Roux-en-Y hepaticojejunoanastomosis

n	Age/sex	Cause of HJA	Success of diagnostic ERC	Finding on diagnostic ERC	No. of ERC	Characteristics and No. of endoscopic therapeutic procedures
1	49/F	ILC after LCE	Yes	HJA stenosis	3	BD-3x, EBD-2x
2	64/F	Condition after RHP (CHP)	Yes	HJA stenosis	1	BD-1x
3	26/F	Congenital malformation choledochus (CBD)	Yes	Cystic dilatation choledochus (CBD)	1	Surgical solution
4	60/M	ILC after LCE	No	HJA found but not probed into	1 (SBE)	Solved by PTC and PTD
5	67/M	ILC after LCE	Yes	CDL	1	ECDL-1x
6	57/F	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x
7	52/M	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x
8	29/M	Condition after OLT (PSC)	Yes	HJA stenosis	3	BD-2x, EBD-2x
9	64/M	ILC after LCE	Yes	Normal ERC results	1	Without endoscopic treatment
10	48/F	ILC after LCE	Yes	HJA stenosis + CDL	2	BD-1x, ECDL-1x, EBD-1x
11	56/F	ILC after LCE	Yes	too narrow HJA stenosis	1	Therapeut. ERC impossible, condition solved by PTC and PTD
12	59/F	ILC after LCE	Yes	CDL	1	ECDL-1x
13	63/F	ILC after LCE	No	HJA not found at all	1 (SBE)	Solved by PTC and PTD
14	59/M	ILC after LCE	No	HJA found but not probed into	1 (SBE)	Solved by PTC and PTD
15	54/M	ILC after LCE	Yes	HJA stenosis	2	BD-2x, EBD-1x

BD: Balloon dilation; CDL: Choledocholithiasis; CHP: Chronic pancreatitis; EBD: Endoscopic insertion of plastic biliary drains; ECDL: Extraction of choledocholithiasis; ILC after LCE: Iatrogenic lesion of the common bile duct after laparoscopic cholecystectomy; RHP: Resection of the head of the pancreas; SBE: Single balloon enteroscopy; ERC: Endoscopic retrograde cholangiography; CBD: Common bile duct; M: Male; F: Female; PTC: Percutaneous transhepatic cholangiography; PTD: Percutaneous transhepatic drainage; HJA: Hepaticojejunoanastomosis; PSC: Primary sclerosing cholangitis.

therefore successful.

Patient 6

ERC was performed twice in patient 6 (57-year-old woman) and slight HJA stenosis and choledocholithiasis were found. Endoscopic balloon dilation of the HJA stenosis was performed during the first ERC, followed by endoscopic balloon extraction of the choledocholithiasis. Control ERC showed satisfactory results for the biliary tree. Both the diagnostic and therapeutic ERC were therefore successful.

Patient 7

ERC was performed twice in patient 7 (52-year-old man). The first ERC showed slight HJA stenosis and choledocholithiasis. Balloon dilation of the HJA stenosis was performed first, followed by endoscopic extraction of the choledocholithiasis using an extraction balloon. Control ERC showed satisfactory results in the biliary tree. Both the diagnostic and therapeutic ERC were therefore successful.

Patient 8

Patient 8 (29-year-old man) underwent HJA construction for a condition that developed after OLT for PSC. ERC was performed three times. The first ERC showed a narrow HJA stenosis. Endoscopic balloon dilation of the stenosis was subsequently performed, followed by endoscopic insertion of a 7 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. The second ERC was performed under the same conditions 1 mo later. No complications occurred during that month. First, a 7 Fr biliary drain was endoscopically extracted. After that, control ERC was performed, showing that the original HJA stenosis was less prominent. Repeated endoscopic balloon dilation of the HJA stenosis was performed. At the end of the second ERC session, a 7 Fr plastic biliary drain was inserted into the bile duct. Third, control ERC was performed under the same conditions 6 wk later. A 7 Fr biliary drain was endoscopically extracted and control ERC was performed, showing significant improvement of the HJA stenosis, which was in fact unnoticeable. Both the diagnostic and therapeutic ERC in this patient were therefore successful.

Patient 9

Successful diagnostic ERC was performed once in patient 9 (64-year-old man) and showed normal findings.

Patient 10

ERC was performed twice in patient 10 (48-year-old woman). The first ERC showed HJA stenosis and slight choledocholithiasis. Balloon dilation of the HJA stenosis was performed first, followed by endoscopic extraction of the choledocholithiasis using an extraction balloon. A 7 Fr plastic biliary drain was subsequently inserted into the bile duct, completely bridging the HJA stenosis. Control ERC in 4 wk (*i.e.*, during the second ERC session) showed satisfactory findings in the bile duct. Both the diagnostic and therapeutic ERC were therefore successful.

Patient 11

ERC was performed once in patient 11 (56-year-old woman) and prominent HJA stenosis was found. Therapeutic ERC, that is, insertion of a balloon or biliary drain into the HJA stenosis, was not possible because the stenosis was too narrow. This led to PTC and PTD. Successful diagnostic ERC was therefore performed once in this patient.

Table 2 Diagnostic endoscopic retrograde cholangiography in patients with Roux-en-Y hepaticojejunoanastomosis

Results in ERC	No. of patients
Normal results	1
Cystic dilation of the bile duct	1
HJA stenosis	5
Choledocholithiasis	2
HJA stenosis + choledocholithiasis	3
Total	12

Cannulation success was reached in 12 of 15 patients [80% diagnostic endoscopic retrograde cholangiography (ERC) success rate]. HJA: Hepaticojejunoanastomosis.

Patient 12

ERC was performed once in patient 12 (59-year-old woman) and slight choledocholithiasis was found. Endoscopic extraction of the choledocholithiasis using an extraction balloon was subsequently performed. Both the diagnostic and therapeutic ERC were therefore successful.

Patient 13

The area of the distal end of the afferent intestinal loop was reached by single balloon enteroscopy in patient 13 (63-year-old woman), nevertheless the HJA was not found. ERC was therefore not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and PTD.

Patient 14

The orifice of the HJA was found in patient 14 (59-year-old woman), nevertheless, the attempt to probe the orifice was not successful. ERC was therefore not performed. This was a case of cannulation failure, thus, the condition had to be resolved by PTC and PTD.

Patient 15

ERC was performed once in patient 15 (54-year-old man). The first ERC showed HJA stenosis. Endoscopic balloon dilation of the stenosis was performed, followed by endoscopic insertion of a 7 Fr plastic biliary drain into the bile duct, which completely bridged the HJA stenosis. The second ERC was performed under the same conditions 1 mo later. No complications occurred during that month. First, a 7 Fr biliary drain was endoscopically extracted. After that, control ERC was performed, showing marked improvement, with the original HJA stenosis being almost unnoticeable. In spite of that, endoscopic balloon dilation of the area of the HJA stenosis was performed. At the end of the second ERC session, control ERC was performed, showing almost normal findings, which means that the original HJA stenosis was no longer noticeable. Both the diagnostic and therapeutic ERC in this patient were therefore successful.

Summary of results

The results clearly show that cannulation was successful in diagnostic ERC in 12 of 15 patients (80% diagnostic success rate). The diagnostic ERC findings in these patients are shown in Table 2. Normal ERC findings were present in one of the 12 patients. ERC in the remaining 11 patients showed some pathological findings. One of these cases (cystic dilation of the bile duct) was subsequently resolved by surgery.

Endoscopic treatment was started immediately after diagnostic ERC in the remaining 10 patients (5 with HJA stenosis, 2 with choledocholithiasis, and 3 with both conditions). This treatment was successful in nine patients (90% therapeutic success rate). Therapeutic ERC was not successful in the other patient due to an extremely narrow HJA stenosis. This condition was resolved by PTC and PTD.

Endoscopic therapeutic procedures were performed as follows. Balloon dilatation of HJA stenosis was performed a total of 11 times in seven patients; choledocholithiasis extraction was performed a total of five times in five patients; plastic biliary stent insertion was performed a total of six times in four patients; and removal of a biliary stent inserted by our team was performed a total of six times in four patients.

Some of these endoscopic procedures are presented in Figures 2 and 3.

Cannulation failure was recorded in three of 15 patients. Causes of failure were: one patient in whom HJA was not found at all; and two cases in which HJA was found but not probed. All three cases were resolved using PTC and PTD.

There were no complications in our group of 15 patients.

Problems with ERC

It is also important to draw attention to some of the pitfalls that we encountered and resolved when performing ERC. (1) Fixing an overtube closely in front of the anastomosis in the area of an enteroenteroanastomosis is not recommended because the overtube, in case of the entrance to the afferent intestinal tube being at an acute angle, made insertion of the enteroscope harder; (2) The afferent intestinal loop could be identified by finding its blind end, and bilioenteral anastomosis can be found a few (5-6) centimeters before this blind end; (3) We suggest using endoscopic accessorium of diameter no larger than 7 Fr in the working channel of the single balloon enteroscope (2.8 cm diameter). Based on our personal experience, the use of an 8.5 Fr dilator and biliary drain, or their insertion via the working channel, was difficult. A 7 Fr dilator was much easier to use for dilation and was followed by the use of a dilation balloon. Biliary drainage was, in indicated cases, easier to perform using two 7 Fr drains; and (4) It is advised to straighten the overtube during extraction of the enteroscope from the overtube by manipulation under skiascopic control but, at the same time, it is necessary to prevent the creation of curves that are small in diameter because they tend to break after the removal of the enteroscope, and make repeated insertion complicated.

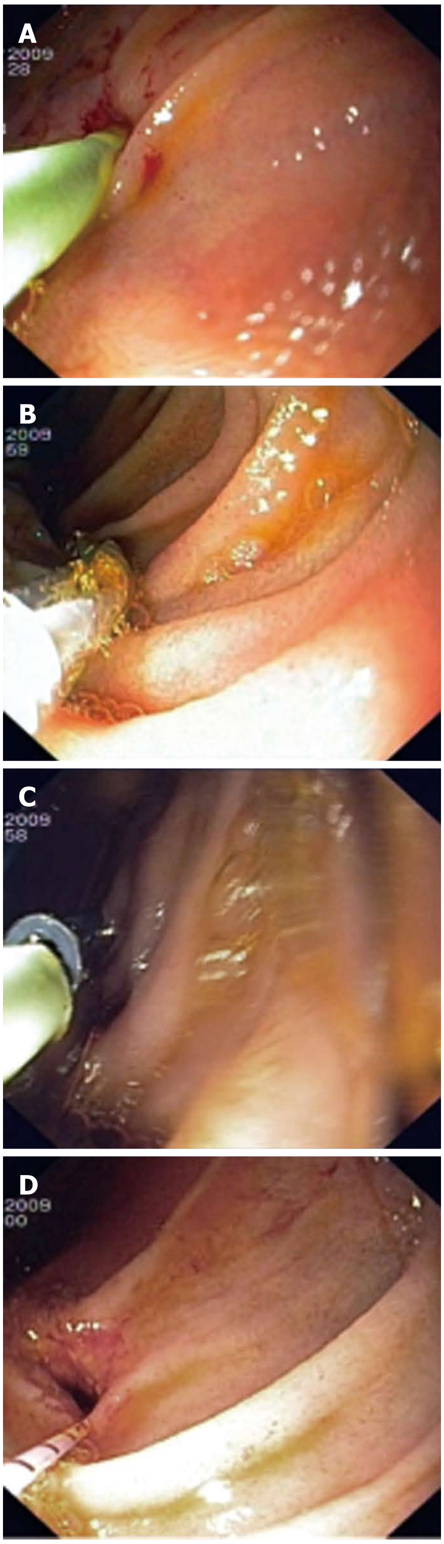

Figure 2 Endoscopic image. A: Successful cannulation of a stenotic orifice of the hepaticojejunoanastomosis (HJA) (followed by successful cannulation of adjoining bile ducts); B: Dilation balloon in a deflated form successfully inserted down the guide wire into the area of the stenotic HJA; C: Last phase of a successful endoscopic insertion of a 7 Fr plastic biliary drain into the stenotic HJA and adjoining bile ducts; D: Highly satisfactory final effect of endoscopic treatment of the stenotic HJA (with inserted guide wire).

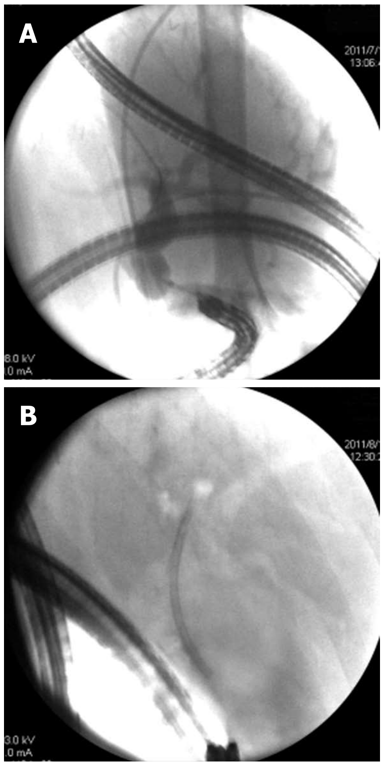

Figure 3 Endoscopic retrograde cholangiography performed by using single balloon enteroscopy. A: A patient with hepaticojejunoanastomosis (HJA) shows comparatively narrow HJA stenosis with suprastenotic dilation of bile ducts; B: The patient from Figure 3A in whom HJA stenosis was successfully bridged by endoscopic insertion of a 7 Fr plastic biliary stent.

DISCUSSION

Single or double balloon enteroscopy enables us to reach even the more distant parts of the small intestine. It is therefore reasonable that this examination method began to be used also for reaching the orifice of the bile duct under conditions of altered anatomy after surgical procedures, when the bile ducts become unreachable by standard lateroscopy (*i.e.*, using conventional ERCP). Previously, pathological conditions (or biliary obstruction) had to be resolved surgically or using PTC[14].

One of these most frequently resolved problems is bile duct pathology in patients with Roux-en-Y HJA (HJA stenosis, choledocholithiasis in the bile duct above the HJA, or both). If we know the type of surgical procedure, ERC using single balloon enteroscopy can be attempted in these cases[14]. Balloon enteroscopy can enable one to reach the afferent intestinal loop, identify the orifice of the HJA[15-17], perform diagnostic ERC, and if indicated, also perform therapeutic ERC. Originally, the procedure was carried out with double balloon enteroscopy[7,18] and later also with single balloon enteroscopy[13,19-21]. There was a wide range of endoscopic therapeutic procedures performed. These procedures are also being performed in standard ERCP[11]. We used single balloon enteroscopy for diagnostic and therapeutic ERC in our group of 15 patients with Roux-en-Y HJA.

The results of diagnostic and therapeutic ERC using single balloon enteroscopy in patients with Roux-en-Y HJA in other endoscopic centers show that Dellon *et al*[20] were successful when performing ERC in three of four patients. Neumann *et al*[21] worked with 13 patients in

whom the diagnostic success rate was 62% and the therapeutic success rate 54%. The largest study to date was by Saleem *et al*[12] who achieved a success rate of 78% (32 of 41 patients) in diagnostic ERC. Wang *et al*[13] have recently described a group of 13 patients (16 procedures in total). Cannulation success rate was 81% (13 of 16 cases) for diagnostic ERC. Therapeutic ERC was necessary in 10 of these 13 patients and was successful in nine patients (90% therapeutic ERC success rate).

The diagnostic and therapeutic ERC success rates in our study were comparatively high and, at the same time, there were no procedure-related complications. Cannulation success was achieved in 12 of 15 patients (80% diagnostic ERC success rate). Endoscopic treatment was successful in nine of 10 patients (90% therapeutic ERC success rate). Our results in diagnostic and therapeutic ERC are comparable to those of other endoscopic centers dealing with this issue[12,13,20].

As mentioned above, we encountered some pitfalls when performing ERC, which are not addressed in detail by other authors[7,12,19,20]. Nevertheless, they do consider the following: (1) avoidance of fixing an overtube closely in front of an enteroenteroanastomosis at the entrance of the enteroscope into the afferent loop; (2) identification of the afferent loop and the bilioenteral anastomosis; (3) using an endoscopic accessorium of diameter no larger than 7 Fr in the working channel of the single balloon enteroscope (diameter 2.8 cm) - a suggestion based on our own experience; and (4) straightening the overtube during extraction of the enteroscope from the overtube, by manipulation under skiascopic control, and at the same time, preventing creation of curves that are small in diameter, because they tend to break after removal of the enteroscope and make repeated insertion complicated.

No complications, not even acute pancreatitis, appeared in our group of 15 patients. It might be that the altered anatomy of the gastrointestinal tract decreases the risk of complications associated with balloon enteroscopy[7].

In conclusion, it can be stated that ERC using single balloon enteroscopy in patients with Roux-en-Y HJA is more difficult than standard ERCP, due to altered postoperative anatomy, and considerable endoscopic skill and experience is needed in order to perform ERC successfully. CRE requires a lot of time for individual procedures and the presence of an anesthesiologist is essential. The cannulation success rate reached in our group of patients was 80% (12 of 15 patients). Endoscopic treatment was successful in 90% (9 of 10 patients). Most ERC procedures in our group of patients were therapeutic (10 of 12 patients - *i.e.*, 83%). There were no complications in our patients. This method is highly demanding but, at the same time, effective and safe, significantly widening the possibilities of resolving biliary tract diseases.

COMMENTS

Background

Single balloon enteroscopy (SBE) was originally and still is used for endoscopic diagnosis and treatment of diseases of the small intestine. It was also introduced in endoscopic retrograde cholangiography (ERC) in patients with Roux-en-Y hepaticojejunoanastomosis (HJA), especially in cases in which standard ERC (by lateroscopy) was unsuccessful, because it was impossible to reach the area of the HJA.

Research frontiers

The need to continue developing and possibly improving equipment (both endoscopes and endoscopic accessories) is still present. They should be better adjusted to the needs of the ERC procedures in patients with Roux-en-Y HJA (enteroscopes with sideways or oblique optics with an elevator, shorter enteroscopes with wider channels specially designed for these procedures, and development of endoscopic accessories of greater length).

Innovations and breakthroughs

When surgically altered gastrointestinal or pancreatobiliary anatomy is present, endoscopic retrograde cholangiopancreatography becomes even more demanding than in a normal anatomical situation. In spite of that, we managed to achieve a comparatively high success in diagnostic (80%) and therapeutic (90%) ERC using single balloon enteroscopy in our cohort of 15 patients with Roux-en-Y HJA, and there were no complications after ERC.

Applications

ERC using single balloon enteroscopy in patients with Roux-en-Y HJA is time consuming and technically demanding. Nevertheless, it is also an effective and safe method that widens the possibilities of resolving biliary tract diseases. Previously, the only possibility of resolution was using percutaneous transhepatic cholangiography or a surgical approach.

Peer review

The study reported high-quality results of diagnostic and therapeutic ERC using single balloon enteroscopy in patients with Roux-en-Y HJA. The study retrospectively evaluated 15 patients with Roux-en-Y HJA with signs of biliary obstruction. Altogether, 23 ERC procedures were performed without any complications.

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BRIEF ARTICLE

Simultaneous follow-up of mouse colon lesions by colonoscopy and endoluminal ultrasound biomicroscopy

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Abstract

AIM: To evaluate the potential use of colonoscopy and endoluminal ultrasonic biomicroscopy (eUBM) to track the progression of mouse colonic lesions.

METHODS: Ten mice were treated with a single azoxymethane intraperitoneal injection (week 1) followed by seven days of a dextran sulfate sodium treatment in their drinking water (week 2) to induce inflammation-associated colon tumors. eUBM was performed simultaneously with colonoscopy at weeks 13, 17-20 and 21. A 3.6-F diameter 40 MHz mini-probe catheter was used for eUBM imaging. The ultrasound mini-probe catheter was inserted into the accessory channel of a pediatric flexible bronchofiberscope, allowing simultaneous acquisition of colonoscopic and eUBM images. During image acquisition, the mice were anesthetized with isoflurane and kept in a supine position over a stainless steel heated surgical waterbed at 37 ℃. Both eUBM and colonoscopic images were captured and stored when a lesion was detected by colonoscopy or when the eUBM image revealed a modified colon wall anatomy. During the procedure, the colon was irrigated with water that was injected through a flush port on the mini-probe catheter and that acted as the ultrasound coupling medium between the transducer and the colon wall. Once the acquisition of the last eUBM/colonoscopy section for each animal was completed, the colons were fixed, paraffin-embedded, and stained with hematoxylin and eosin. Colon images acquired at the first time-point for each mouse were compared with subsequent eUBM/colonoscopic images of the same sites obtained in the following acquisitions to evaluate lesion progression.

RESULTS: All 10 mice had eUBM and colonoscopic images acquired at week 13 (the first time-point). Two animals died immediately after the first imaging acquisition and, consequently, only 8 mice were subjected to the second eUBM/colonoscopy imaging acquisition (at the second time-point). Due to the advanced stage of colonic tumorigenesis, 5 animals died after the second time-point image acquisition, and thus, only three were subjected to the third eUBM/colonoscopy imaging acquisition (the third time-point). eUBM was able to detect the four layers in healthy segments of

colon: the mucosa (the first hyperechoic layer moving away from the mini-probe axis), followed by the muscularis mucosae (hypoechoic), the submucosa (the second hyperechoic layer) and the muscularis externa (the second hypoechoic layer). Hypoechoic regions between the mucosa and the muscularis externa layers represented lymphoid infiltrates, as confirmed by the corresponding histological images. Pedunculated tumors were represented by hyperechoic masses in the mucosa layer. Among the lesions that decreased in size between the first and third time-points, one of the lesions changed from a mucosal hyperplasia with ulceration at the top to a mucosal hyperplasia with lymphoid infiltrate and, finally, to small signs of mucosal hyperplasia and lymphoid infiltrate. In this case, while lesion regression and modification were observable in the eUBM images, colonoscopy was only able to detect the lesion at the first and second time-points, without the capacity to demonstrate the presence of lymphoid infiltrate. Regarding the lesions that increased in size, one of them started as a small elevation in the mucosa layer and progressed to a pedunculated tumor. In this case, while eUBM imaging revealed the lesion at the first time-point, colonoscopy was only able to detect it at the second time-point. All colonic lesions (tumors, lymphoid infiltrate and mucosal thickening) were identified by eUBM, while colonoscopy identified just 76% of them. Colonoscopy identified all of the colonic tumors but failed to diagnose lymphoid infiltrates and increased mucosal thickness and failed to differentiate lymphoid infiltrates from small adenomas. During the observation period, most of the lesions (approximately 67%) increased in size, approximately 14% remained unchanged, and 19% regressed.

CONCLUSION: Combining eUBM with colonoscopy improves the diagnosis and the follow-up of mouse colonic lesions, adding transmural assessment of the bowel wall.

Key words: Ultrasound biomicroscopy; Animal model; Diagnostic imaging; Colonic neoplasm; Longitudinal study

Core tip: This paper employed imaging methods, endoluminal ultrasonic biomicroscopy (eUBM) associated to colonoscopy, in a longitudinal study to evaluate the progression of chemically-induced colonic lesions in mice, during a period of two months. The eUBM method complemented colonoscopy and enhanced the study, once the ultrasonic images allowed the detection of lesions underneath the epithelium. Potential future application of eUBM combined with colonoscopy could be in the monitoring of therapeutic efficacy of chemotherapeutic drugs *in vivo*.

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INTRODUCTION

Colorectal cancer (CRC) has a high incidence in the world as it is the third most common cancer in men and women in developed countries[1]. It is estimated that more than 142000 people in the United States will be diagnosed with CRC in 2013[2]. In Europe, CRC is detected in approximately 413000 people each year, half of whom die during the course of the disease. Despite its high incidence and mortality rates, the majority of CRC-related deaths could be prevented through the implementation of powerful tools for CRC early detection and staging.

Currently, colonoscopy is the recommended screening method for CRC screening and follow-up, but it has some limitations. Studies have demonstrated that the detection of adenomas, serrated polyps and sessile serrated adenomas differs significantly among endoscopists[3]. Furthermore, colorectal neoplasms of a diminutive size (smaller than 10 mm) or nonpolypoid shape may be more easily overlooked during a routine colonoscopy[4-6]. The miss rate for CRC lesions may explain the high proportion (3.3%-12.4%) of proximal CRC that is diagnosed shortly after a clearing colonoscopy[7,8]. Therefore, the efforts of some research groups are focused on the development of other imaging methods that complement the results of a colonoscopy.

High frequency endoscopic ultrasonography (EUS) is a relatively new technique in which an ultrasonography probe is inserted into the accessory channel of a regular endoscope. EUS has the capacity to look deep below the lining of the colon and is a useful modality for transmural assessment of the bowel wall[9,10]. Usually, the ultrasound transducers used in EUS instrumentations operate at low frequencies (7.5-12 MHz), but higher ultrasound frequencies are also employed by using a mini-probe[11-13]. Higher ultrasound frequencies increase EUS resolution, allowing for the staging of colon tumors and the visualization of small colonic lesions. The use of high frequency mini-probe ultrasound for the diagnosis of mucosal and submucosal colorectal lesions and for the guidance of lesion resection has already been proposed as a safe and effective technique[13,14]. However, the role for high frequency mini-probe ultrasound in the routine diagnosis of colonic lesions has not been fully established[15-18].

Animal models of diseases can be used to develop and evaluate new diagnostic tools before they are applied clinically. The development of non-invasive experimental imaging modalities allows for the study of the same ani-

mal over time, enabling the investigation of disease development and therapeutic interventions. Mouse models of chemically induced CRC are highly reproducible, can be tested on animals with different genetic backgrounds and recapitulate human CRC. The use of an effective and valuable mouse model of chemically induced CRC can help investigators understand colonic tumorigenesis and to probe novel diagnostic platforms for use in clinical practice[19].

Our group has previously used ultrasonic biomicroscopic (UBM) instrumentation, operating at 45 MHz, for *in vitro* imaging of chemically induced mouse CRC[20] to demonstrate that UBM is a feasible tool to identify the layers of mouse colon with adequate contrast between them and with sufficient resolution. Afterwards, endoluminal UBM (eUBM), operating at 40 MHz, was performed along with a colonoscopy, and simultaneous eUBM and colonoscopic images were generated *in vivo*[21].

Recently, studies have verified the efficacy of UBM as a tool for longitudinal studies in mice: Harmon and co-workers[22] validated the use of 40 MHz extracorporeal UBM for carotid plaque development in mice; Tiwari *et al*[23] used a 40 MHz UBM for longitudinal monitoring of infliximab treatment efficacy in a mouse model of pancreatic cancer; Fernández-Domínguez *et al*[24] also used a 40 MHz UBM in a longitudinal study to evaluate the progression of fatty liver disease in mice; and Campos-Junior *et al*[25] analyzed the efficacy of UBM in the evaluation of induced ovarian follicular growth and ovulation in mice. Despite growing evidence confirming the efficacy of high frequency ultrasound in the monitoring of lesion progression, the ability of eUBM to diagnose colonic tumoral development in animal models has not yet been studied.

The present work comprises the use of eUBM instrumentation associated with colonoscopy in a longitudinal study to evaluate the progression of chemically induced colonic lesions in mice.

MATERIALS AND METHODS

Animals

Ten mice [*Mus musculus* (Linnaeus, 1758)] of both genders, with an average age of 7 wk, an average weight of 25 g, and *p53*$^{+/+}$ and *p53*$^{+/-}$ (heterozygous for tumor suppressor gene *Trp53*), were used. The mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, United States) and kept in the 129/SvJ background. We used the *p53*$^{+/-}$ mice because *Trp53* mutations accelerate tumorigenesis in several tissues, including the colon[26].

The animals were maintained at room temperature with the appropriate circadian cycle and diet. The Guide for Care and Use of Laboratory Animals (National Institutes of Health) was also considered.

Colon tumors were induced using a protocol (DAHE-ICB 042) approved by the Animal Care and Use Committee of the Biological Science Institute/Federal University of Rio de Janeiro. The studies involving colon imaging, such as eUBM combined with colonoscopy, were conducted under a protocol (71/08) approved by the Ethical Committee for Laboratory Animal Research/Federal University of Rio de Janeiro.

Azoxymethane and dextran sulfate sodium carcinogenesis protocol

Inflammation-related colon tumors were induced using azoxymethane (AOM) and dextran sulfate sodium (DSS)[27-29]. AOM is a colon-specific carcinogen that can be combined with DSS, a mucosal-irritant agent, to mimic inflammation-associated colon carcinogenesis[29,30]. The animals were subjected to a single intraperitoneal (*ip*) injection of AOM (A5486; Sigma Aldrich, St. Louis, MO, United States) with a concentration of 12.5 mg/kg. One week after AOM administration, the mice were fed with water containing 3% DSS salt, 36000-50000 Da (02160110; MP Biomedicals, Santa Ana, CA, United States), for 1 wk. All of the mice received solid food and water *ad libitum*, with regular water given after the week of DSS intake.

Endoluminal ultrasonic biomicroscopy system

Briefly, images were generated by employing a 3.6-F diameter 40 MHz mini-probe catheter (Atlantis® SR Pro Coronary Imaging Catheter; Boston Scientific Corporation, Natick, MA, United States) mechanically driven by a motordrive unit (MD5; Boston Scientific Corporation, Natick, MA, United States). The ultrasonic transducer rotates 360° around its axis, providing cross-sectional ultrasound images of the colon wall. More details concerning the eUBM instrumentation are described in Alves *et al*[21].

Simultaneous eUBM and colonoscopic image acquisition

Colonoscopy was used simultaneously with eUBM and served to guide the mini-probe through the colon. The ultrasound mini-probe catheter was inserted into the accessory channel of a pediatric flexible bronchofiberscope (FB120P; Fujinon, Tokyo, Japan), allowing simultaneous acquisition of colonoscopy and eUBM images. The bronchofiberscope has a total length of 920 mm and outer diameters of 2.8 and 2.7 mm for the flexible and distal-end portions, respectively.

To ensure that the colonoscopy and eUBM techniques acquired simultaneous images from the same region, the ultrasonic transducer, at the mini-probe imaging core tip, was positioned outside of the distal end accessory channel extremity, while still as close as possible to the bronchofiberscope extremity. The mini-probe telescoping shaft section was used to advance and retract the imaging core, placing it in the correct position.

During image acquisition, the mice were anesthetized with isoflurane (Cristália; São Paulo, Brazil) at 1.5% in 1.5 L/min oxygen, using a laboratory animal anesthe-

Figure 1 Schematic overview of the azoxymethane and dextran sulfate sodium model and subsequent image acquisition. A single azoxymethane (AOM) *ip* injection was given to 6-wk-old mice (week 1). One week later (week 2), 3% dextran sulfate sodium (DSS) administration was given in the drinking water for 7 d, followed by regular water. The first endoluminal ultrasonic biomicroscopy (eUBM) and colonoscopic images were acquired at week 13, the second acquisition was from weeks 17-20, and the third acquisition was at week 21.

sia system (EZ-7000; Euthanex, Palmer, PA, United States). The animals were kept in a supine position over a stainless steel heated surgical waterbed at 37 ℃ using the T/Pump System (Gaymar, Orchard Park, NY, United States). Before the examination, an enema was performed with 1 mL of water to remove feces. Subsequently, the flexible bronchofiberscope containing the ultrasound mini-probe catheter was introduced into the descending colon. Both eUBM and colonoscopy images were captured simultaneously and stored when a lesion was detected by colonoscopy or when the eUBM image revealed a modified colon wall anatomy. During the procedure, the colon was irrigated with water that was injected through a flush port of the mini-probe catheter and that acted as the ultrasound coupling medium between the transducer and the colon wall.

Study design

The sequential evaluation of colonic lesions by simultaneous *in vivo* eUBM and colonoscopic imaging started at 13 wk after AOM administration and was performed at three different time-points, according to Figure 1: the first one at week 13, the second one between weeks 17 and 20 and the last one at week 21.

Colon lesion images acquired at the first time-point for each mouse were compared with subsequent eUBM/colonoscopic images of the same sites obtained in the following acquisitions. After the last eUBM examination, the images of each lesion were separated for subsequent comparison with histopathology.

Histological analysis

Once the acquisition of the last image for each animal was completed, each anesthetized mouse was euthanized by cervical dislocation. The distal colon was excised, cleaned and fixed in 4% formaldehyde for 16 h before paraffin embedding. The paraffin-embedded tissues were cross-sectioned (5 μm) stepwise transversally to the colon longitudinal axis and stained with hematoxylin and eosin. All stained sections were analyzed by light microscopy and compared with the ultrasonic images, whose frames were obtained from the same lesions observed with the eUBM and/or colonoscopy.

Table 1 Simultaneous endoluminal ultrasonic biomicroscopy and colonoscopy image acquisition on colon tumor-bearing mice

	Weeks after AOM administration		
Mouse number	1st eUBM	2nd eUBM	3rd eUBM
1	13	-	-
2	13	-	-
3	13	18	-
4	13	20	-
5	13	20	-
6	13	20	-
7	13	20	-
8	13	17	21
9	13	17	21
10	13	17	21

eUBM: Endoluminal ultrasonic biomicroscopy; AOM: Azoxymethane.

RESULTS

The time-points for image acquisition of each animal are presented in Table 1. All 10 mice had eUBM and colonoscopic images acquired at week 13. Two animals died immediately after the first imaging acquisition and, consequently, only eight mice were subjected to the second eUBM/colonoscopy imaging acquisition. Due to the advanced stage of colonic tumorigenesis, five animals died after the second time-point image acquisition, and thus, only three were subjected to the third eUBM/colonoscopy imaging acquisition.

An example of interrelated eUBM and histological images of a healthy section from the mouse colon is presented in Figure 2A. The mucosal layer is seen as a hyperechoic circular layer (the first hyperechoic layer moving away from the mini-probe axis), followed by a hypoechoic layer representing the muscularis mucosae. The submucosa corresponds to a hyperechoic layer, followed by the muscularis externa, the second hypoechoic layer. At the center of the lumen is the ultrasound mini-probe, represented by a gray circle. An eUBM image of a colonic lymphoid infiltrate, represented by a hypoechoic region between the mucosa and muscularis externa layers, is presented in Figure 2B with the corresponding histological image.

An example of an eUBM image of a pedunculated tumor, whose size increased during the 6 wk between the first and second time-points, is presented in Figure 3A. At the first eUBM exam, a small elevation in the mucosa layer is seen, indicating an early adenoma. At this time, colonoscopy was unable to visualize the lesion. Six weeks later, eUBM showed that the adenoma had increased in size, and the lesion was then observed in the colonoscopic image. Figure 3B presents an eUBM image of a pedunculated adenoma, whose size remained virtually unchanged between the first and third image acquisitions. The adenoma was also visualized in all of the colonoscopy sections. Finally, a sequence of three eUBM images of a lesion that decreased in size during the observation period is depicted in Figure 3C. This lesion was identified at the first eUBM exam (Figure

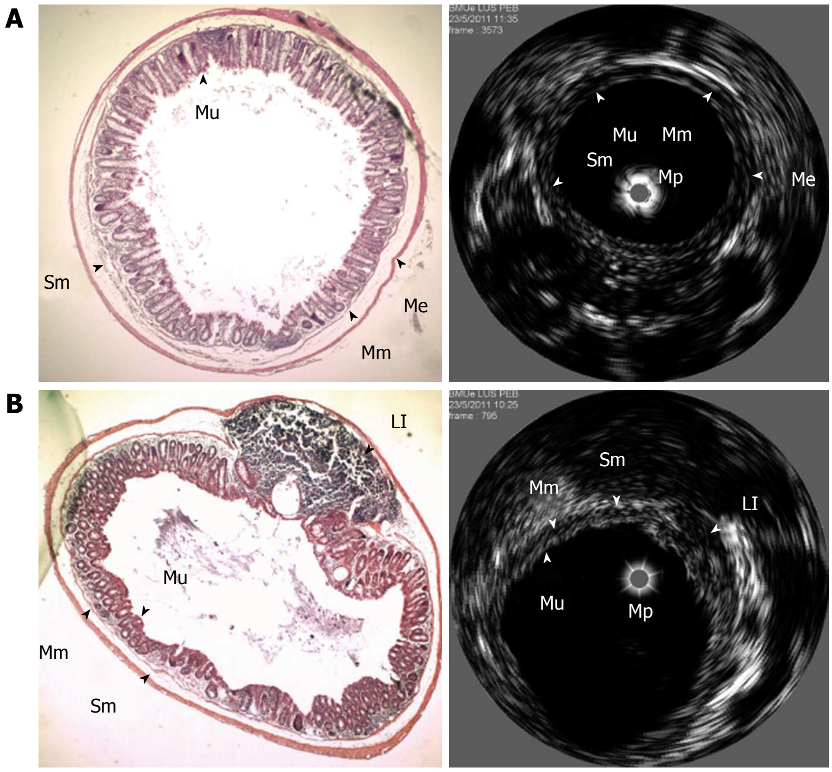

Figure 2 Correlation between endoluminal ultrasonic biomicroscopy and histological images. A: Endoluminal ultrasonic biomicroscopy (eUBM) (right) and the corresponding hematoxylin and eosin-stained histological section (left, × 40 magnification) obtained from a healthy region of a mouse colon. The eUBM image displays two hyperechoic layers: mucosa (Mu) and submucosa (Sm) and two hypoechoic layers: muscularis mucosae (Mm) and muscularis externa (Me). The ultrasound catheter mini-probe (Mp) is at the center of the lumen; B: eUBM (right) and the corresponding hematoxylin and eosin-stained histological section (left, × 40 magnification) obtained from a mouse colon containing a lymphoid infiltrate in the colonic wall. The eUBM image displays the mucosa (Mu), muscularis mucosae (Mm) and submucosa layer (Sm). The lymphoid infiltrate (LI) lesion is seen as a hypoechoic region underneath the mucosa. The ultrasound catheter mini-probe (Mp) is at the center. All layers identified in the ultrasound images are well correlated with the histological images from the same site.

3C-a) as a mucosal hyperplasia with ulceration at the top. The ulceration was also visualized by colonoscopy. Four weeks later, at the second eUBM exam (Figure 3C-b), the mucosal hyperplasia had decreased, and a hypoechoic area underneath the mucosa was observed, indicating the emergence of an inflammatory infiltrate. At this point, colonoscopy showed no alterations in this colonic section. At the last eUBM exam (Figure 3C-c), both the mucosal hyperplasia and lymphoid infiltrate had almost completely disappeared. Histological analysis of the same section confirmed the presence of the remaining diminutive lymphoid infiltrate section (Figure 3C-d).

Colonic lesions detected by either the last eUBM or colonoscopy, and confirmed by *post mortem* histology, are indicated in Table 2. Altogether, eUBM identified all of the lesions (tumors, lymphoid infiltrate and mucosal thickening), while colonoscopy identified just 76% of them. Colonoscopy identified all colonic tumors but failed to diagnose lymphoid infiltrates and increased mucosal thickness and failed to differentiate lymphoid infiltrates from small adenomas.

Additionally, the lesion progression outcomes, based on eUBM image analysis, are presented in Table 2. During the observation period, most of the lesions (approximately 67%) increased in size, approximately 14% remained unchanged and 19% regressed.

DISCUSSION

This report describes the use of a eUBM imaging system for the detection and follow-up of mouse colonic lesions. The simultaneous use of eUBM with colonoscopy was able to detect, diagnose and analyze the progression of tumoral and non-tumoral lesions in a CRC mouse model. Our group has previously demonstrated that two UBM systems, one operating at 45 MHz and the other at 40 MHz, could diagnose mouse colonic lesions *in vitro*[20] and *in vivo*[21], respectively. Here, we have demonstrated that a variety of colon lesions can be detected by eUBM in a minimally invasive way. In contrast to histopathological analysis, eUBM can be employed to make repeated measures on the same animal, facilitating the investigation of pathological processes and therapies.

Similar to the previous work, the ultrasound images obtained with eUBM also allowed for the visualization of normal colonic layers: the mucosa, muscularis mucosae, submucosa and muscularis externa (Figure 2A), as well as colon alterations, such as lymphoid infiltrates, ulcerations and tumors (Figure 2B and Figure 3). Confirming our previous findings, lymphoid infiltrates appear as hypoechoic regions underneath a hyperechoic layer representing the mucosa. Colon tumors appear as hyperechoic masses above the mucosa layer. This characteriza-

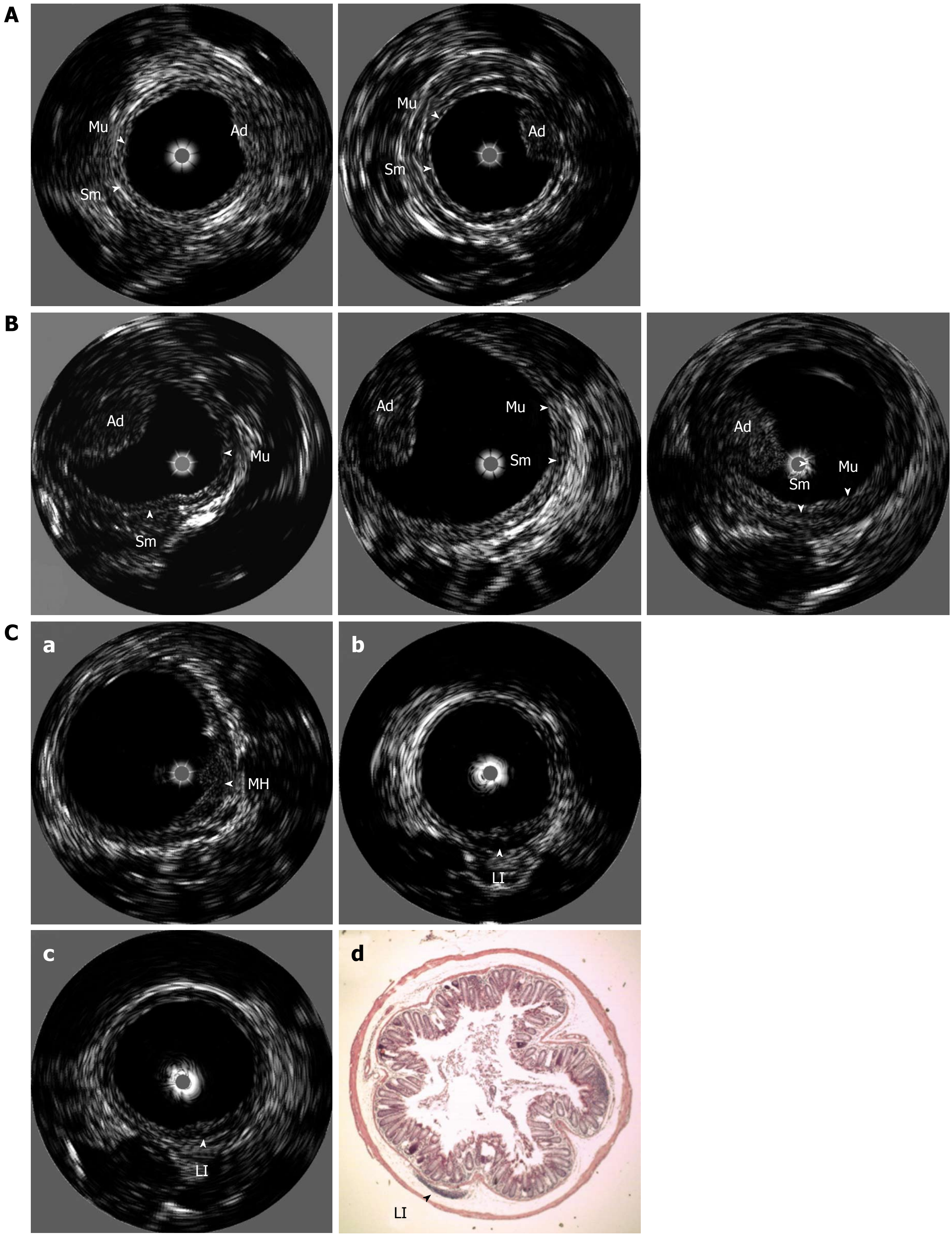

Figure 3 Endoluminal ultrasonic biomicroscopy images. A: Show increase in tumor volume. Endoluminal ultrasonic biomicroscopy (eUBM) colon images acquired at the first (left) and second (right) time-points from azoxymethane (AOM)-dextran sulfate sodium (DSS)-treated mice. The volume of the pedunculated adenoma (Ad) increased between the first and second eUBM examinations; B: Show no alteration in tumor volume. eUBM colon images acquired at the first (left), second (middle) and third (right) time-points from AOM-DSS-treated mice. The lesion observed is a pedunculated tumor. Images show that the tumor volume is unchanged during the observation period; C: Show reduction in lesion size. eUBM colon images acquired at the first (a), second (b) and third (c) time-points from AOM-DSS-treated mice. The lesion observed at the first eUBM image is a mucosa hyperplasia (MH) with ulceration at the top. In the subsequent eUBM image, the MH has decreased, and a lymphoid infiltrate (LI) has appeared in the submucosa layer. In the third and last eUBM image, MH and LI have almost completely disappeared, which is confirmed by histological analysis (d, × 40 magnification). Mu: Mucosa; Sm: Submucosa.

tion is of great importance because it could be used to distinguish small adenomatous polyps from lymphoid

Table 2 Lesion progression observed by longitudinal endoluminal ultrasonic biomicroscopy and colonoscopic imaging

Animals	Animal lesion	Lesion detection						Lesion progression					
		eUBM			Colonoscopy			Size			Lesion type		
		N°	Yes	No	Yes	No	Obs	↑	↓	=	Tu	LI	MT
1	L1-1	1	√		√			√			√	√	
2	L1-2	1	√		√		√	√			√		
3	L1-3	2	√		√			√			√		
	L2-3		√		√			√			√		
4	L1-4	2	√		√			√			√		
	L2-4		√		√					√		√	
	L3-4		√		√			√				√	
	L4-4		√					√					√
5	L1-5	2	√		√			√			√		
	L2-5		√				√	√				√	
6	L1-6	2	√		√			√			√		
	L2-6		√		√			√				√	
	L3-6		√		√					√	√		
	L4-6		√			√				√		√	
7	L1-7	2	√			√			√			√	
	L2-7		√				√	√			√		
8	L1-8	3	√		√				√		√		
	L2-8		√		√			√			√		
	L3-8		√			√				√		√	
9	L1-9	3	√						√			√	√
10	L1-10	3	√		√			√			√		

Tu: Tumor; LI: Lymphoid infiltrate; MT: Mucosal thickening; eUBM: Endoluminal ultrasonic biomicroscopy; Obs: impossible to analyze due to colonic hemorrhage or feces. ↑: Increased lesion size; ↓: Decreased lesion size; =: No alteration.

hyperplasias, both seen by colonoscopy as mucosal elevations.

The correct detection and diagnosis of colonic neoplasias during a colonoscopy is essential for CRC prevention. The ranges for adenoma detection rates during a routine colonoscopy could vary up to 37% among endoscopists[3], increasing the chances to misdiagnose CRC. Most postcolonoscopy cancers have a small macroscopic appearance[31,32] and in these cases, the simultaneous use of eUBM with colonoscopy could aid in accurately detecting submucosal invasion in colonic lesions.

The small elevation in the mucosa layer observed with eUBM and registered in Figure 3A was not detected by colonoscopy. Perhaps, this fact was due to the poor bronchofiberscope image quality and could be overcome with high-resolution scopes designed specifically for work with rat and mouse models of colonic diseases[33]. These high-resolution scopes are usually rigid telescopes and a working channel is formed in a space between an operating sheath and the telescope external wall. Although the bronchofiberscope used in the present work is unable to produce high-resolution images, it has the advantage of being flexible. According to the authors' experience, this facility of the bronchofiberscope is important to position the eUBM mini-probe tip close to a lesion, which improves the lesion visualization, or at the center of the colon lumen in order to generate circular eUBM images of the colon.

According to the results obtained with this longitudinal evaluation of inflammation-associated colon tumor progression, most of the lesions increased in size, mimicking human cancer development. All tumoral lesions were diagnosed at the first analysis (13 wk after AOM administration), even when the size was very diminutive. The diameter of the smallest detected tumoral lesion was 0.45 mm, and eUBM was able to identify even smaller structures, such as mucosal elevations with a height of 0.1 mm. Of all the lesions detected by eUBM, approximately 15% of them (four lesions) showed a reduction in size. Of these lesions, two were lymphoid infiltrates, whose size reduction indicated inflammation resolution; one was an increase in mucosal thickness that regressed; and the last one was a small adenoma whose tumoral mass decreased. Besides pedunculated and depressed lesions, we have also detected flat lesions in animal models of CRC and current work is being conducted using *p53* knockout mice, which develop flat lesions with a higher incidence than wild type mice[34], to evaluate the eUBM sensitivity.

The data presented here suggest that the use of high-resolution endoluminal ultrasound is a valuable tool to evaluate the progression of colonic lesions. eUBM detected alterations in mouse colonic lesion morphology and in adenoma volume throughout the examination period. Longitudinal high-resolution ultrasound measurements could be helpful in the monitoring of therapeutic efficacy of chemotherapeutic drugs *in vivo*. Additionally, this technique allows for the study of lesion progression in animal models, providing detailed insights into the biology of tumor development.

The potential of the eUBM technique to differentiate malignant from non-malignant lesions is yet to be implemented. Nowadays, the technique of narrow band imaging (NBI) has the capacity to diagnose colon lesion malignancy in real time based in mucosal and superficial vascular structures imaging enhancement[35]. However, eUBM has the potential to detect lesion penetration depth through submucosal layers. Both methodologies have their advantages and limitations and could be performed simultaneously to complement each other.

Another advantage of longitudinal eUBM imaging is the possibility to use ultrasound contrast agents to target specific molecules involved in tumor development, such as the angiogenic promoter vascular endothelial growth factor, providing a minimally invasive tool for molecular diagnosis. This new modality of molecular imaging is now being tested in preclinical models with successful results in the characterization of tumor response to anti-angiogenic treatment[36-38].

In summary, the simultaneous use of eUBM with colonoscopy enhances the ability to correctly diagnose and follow-up colonic lesions, offering rapid imaging acquisition and distinct advantages because high-resolution transmural imaging of the bowel wall improves lesion detection and cost-effectiveness.

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COMMENTS

Background

Colonoscopy is the recommended screening method for colorectal cancer screening and follow-up, but it fails to detect some small or nonpolypoid lesions. Therefore, the development of other imaging methods that complement the results of colonoscopy is extremely important. The authors have previously show that endoluminal ultrasonic biomicroscopy (eUBM) associated to colonoscopy improves the detection and diagnose of inflammatory and tumoral colonic lesions in animal models. Here the authors analyze the capacity of eUBM to evaluate the progression of chemically-induced colonic lesions in mice.

Research frontiers

The use of eUBM to diagnose mucosal and submucosal colorectal lesions and to guide lesion resection has already been proposed as a safe and effective clinical technique. However, its significant role in the routine diagnosis of colonic lesions has not yet been established. The use of animal models contributes in the development and evaluation of new diagnostic tools before they are completely clinically applied.

Innovations and breakthroughs

A step forward of previous work done by our group, which now includes the longitudinal study of lesion progression.

Applications

The current results suggest that the use of eUBM simultaneously to colonoscopy enhances the ability to correctly diagnose and follow up colonic lesions. In addition to its potential clinical application, eUBM can aid investigators to study colonic tumorigenesis processes and to evaluate novel therapeutic agents for colorectal cancer.

Terminology

eUBM, also known as high frequency endoscopic ultrasonography is a relatively new technique in which an ultrasound probe is inserted into the accessory channel of a regular endoscope. eUBM is an useful modality for transmural assessment of the bowel wall.

Peer review

The authors describe the evaluation of the potential use of colonoscopy and eUBM to track the progression of mouse colonic lesions. This is a clinically very interesting study.

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BRIEF ARTICLE

Effects of disease severity and necrosis on pancreatic dysfunction after acute pancreatitis

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Abstract

AIM: To evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis (AP).

METHODS: One hundred and nine patients treated as AP between March 2003 and September 2007 with at least 6 mo follow-up were included. Patients were classified according to severity of the disease, necrosis ratio and localization. Subjective clinical evaluation and fecal pancreatic elastase-Ⅰ (FPE-Ⅰ) were used for exocrine dysfunction evaluation, and oral glucose tolerance test was completed for endocrine dysfunction. The correlation of disease severity, necrosis ratio and localization with exocrine and endocrine dysfunction were investigated.

RESULTS: There were 58 male and 51 female patients, and mean age was 56.5 ± 15.7. Of the patients, 35.8% had severe AP (SAP) and 27.5% had pancreatic necrosis. Exocrine dysfunction was identified in 13.7% of the patients [17.9% were in SAP, 11.4% were in mild AP (MAP)] and 34.7% of all of the patients had endocrine dysfunction (56.4% in SAP and 23.2% in MAP). In patients with SAP and necrotizing AP (NAP), FPE-Ⅰ levels were lower than the others (P < 0.05 and 0.001 respectively) and in patients having pancreatic head necrosis or near total necrosis, FPE-1 levels were lower than 200 μg/g stool. Forty percent of the patients who had undergone necrosectomy developed exocrine dysfunction. Endocrine dysfunction was more significant in patients with SAP and NAP (P < 0.001). All of the patients in the necrosectomy group had endocrine dysfunction.

CONCLUSION: Patients with SAP, NAP, pancreatic head necrosis and necrosectomy should be followed for pancreatic functions.

Key words: Acute pancreatitis; Exocrine dysfunction; Endocrine dysfunction; Pancreas function test; Pancreatic necrosis

Core tip: The aim of this study was to evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis (AP). Exocrine and endocrne dysfunctions were investigated according to disease severity and necrosis ratio after acute pancreatitis. Exocrine dysfunction was identified in 13.7% of the patients [17.9% were in severe AP (SAP), 11.4% were in mild AP (MAP) and 34.7% of all of the patients had endocrine dysfunction (56.4% in SAP and 23.2% in MAP)]. Forty percent of the patients who had undergone necrosectomy developed exocrine dysfunction. Endocrine dysfunction was more significant in patients with SAP and NAP. All of the patients in the necrosectomy group had endocrine dysfunction. Patients with SAP, NAP, pancreatic head necrosis and necrosectomy should be followed for pancreatic functions.

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INTRODUCTION

Eighty percent of the pancreatic mass is devoted to exocrine function, and the remaining part is responsible for endocrine function, which is crucial to the maintenance of homeostasis of the body[1]. Clinically, the severity of acute pancreatitis (AP) varies significantly. Some patients experience a mild form (mild AP, MAP) of the disease (80%-90% of all cases), which is a self-limiting condition with patients recovering within 3-4 d after onset of the disease. Serious insult occurs in 20%-30% of the cases in the first week after AP attack and mortality can be 30% in the severe form. Pancreatic necrosis develops in 20% of all cases[2,3]. Both the presence and extent of the necrosis affects the clinical course of the disease. Necrosis larger than 50% of the pancreatic mass significantly increases the local and systemic complication rates[4]. There are contradictory results from evaluations of exocrine and endocrine dysfunction in mild and severe cases[5,6]. In 1984 at the Marseille symposium, it was accepted that pancreatic injury is temporary and endocrine and exocrine functions recover during the following month[7]. But there are some contradictory reports claiming that pancreatic injury is persistent[8-10]. Currently, it is accepted that pancreatic function recovers in the absence of pancreatic necrosis and if necrosectomy is not performed[11].

Previous studies have demonstrated that severity of AP, extent of the pancreatic necrosis and the cause of pancreatitis are closely related to the magnitude of pancreatic dysfunction[12,13]. After severe acute pancreatitis (SAP), Bozkurt *et al*[14] and Boreham *et al*[15] reported 85% and 86% pancreatic exocrine dysfunction, respectively. Boreham also reported 13% pancreatic exocrine dysfunction after mild cases. The etiologic factor is also correlated with the level of pancreatic injury; acute pancreatitis due to alcohol consumption may cause pancreatic dysfunction[9]. While it is mostly β cell injury that induces endocrine dysfunction, insulin resistance may also contribute to glucose intolerance[16-18]. Endocrine dysfunction is reported in 15%-35% of the cases[12,14,18].

There is no consensus about the frequency and severity of endocrine and exocrine dysfunction due to acute pancreatitis. Also, experts do not agree on the necessity of enzyme supplementation following the discharge of these patients[15]. Our study aimed to clarify the relationship between pancreatic dysfunction, the severity of the disease and the extent of the pancreatic necrosis.

MATERIALS AND METHODS

This study was undertaken in the Uludag University Department of Surgery-Bursa, Turkey. From March 2003 to October 2007, 216 consecutive patients with AP were evaluated in our center. This study was approved by the Institutional Review Board of Uludag University (September 11th 2007, No: 2007-14/64). Patients who died (n = 16) or with less than 6 mo of follow-up after onset of the disease (n = 11) were excluded. All of the patients were invited to the hospital to participate the study by phone or mail. Fifty-five patients could not be contacted due to change of address and 25 patients declined to participate. The remaining 109 patients were included the study, and written informed consent was obtained from each subject.

The data of the patients who were treated for AP were recorded prospectively in previously prepared forms. Diagnosis of AP and determination of its etiology were based on clinical evaluation, serum and urine amylase (higher than three times the upper level of normal was considered diagnostic), liver function tests, serum triglycerides, calcium, alkaline phosphatase and abdominal ultrasound (US) at the admission. Within 72 h following admission, contrast enhanced abdominal computed tomography (CECT) was performed. Patients with gallstones on US were assumed to be cases of biliary pancreatitis; patients consuming large amounts of alcohol (but not having chronic pancreatitis) were considered as having alcoholic pancreatitis. In patients with high levels of serum fat (triglyceride level more than 1000 mg/dL), hyperlipidemia was accepted as the etiological factor. Patients with undetermined etiology were considered to be idiopathic cases. For prognostic evaluation and classification of the severity of the disease, the Acute and Physiology and Chronic Health Evaluation Ⅱ scoring system (APACHE Ⅱ) was used. Patients with APACHE Ⅱ $\geqslant$ 8 were accepted as severe AP (SAP). While patients who had < 8 score were accepted as mild AP (MAP) and treated conservatively (fluid resuscitation only), patients who had SAP were treated with aggressive fluid resuscitation, nutritional support (enteral or parenteral) and antibiotic prophylaxis. If the patient's clinical status deteriorated and CECT findings revealed infected necrosis or if fine needle aspiration cytology demonstrated infection, they were treated surgically. The Beger procedure (open necrosectomy and closed continuous lavage) plus feeding jejunostomy was our treatment of choice. In some of the cases, more conservative procedures (*i.e.*, percutaneous drainage) had to be undertaken due to the patient's condition.

Following designation of the participating patients and receiving informed consent, specific questions were asked of the patients to evaluate the clinical findings of the pancreatic exocrine insufficiency. The findings were recorded on the Subjective Clinical Evaluation (SCE) form (Table 1). If a patient answered "yes" to even only one of the questions on the SCE form, the test was accepted as positive. Exocrine pancreatic function was also evaluated using the fecal pancreatic elastaz-1 (FPE-1) test in a random stool sample. Patients who were having pancreatic enzyme supplements were instructed to taper their enzyme supplementation 1 mo before the FPE-1 test. Stool samples were collected from the patients and

Table 1 Subjective clinical evaluation test

Age/sex:
- Did DM develop after AP?
(a) yes (b) no
- Any abdominal pain, discomfort, steatorrhea, weakness, weight loss, lack of appetite after AP?
(a) yes (b) no
- Did you use pancreatic enzyme supplementation? If so, how long time did you use it?
(a) yes (b) no

AP: Acute pancreatitis; DM: Diabetes mellitus.

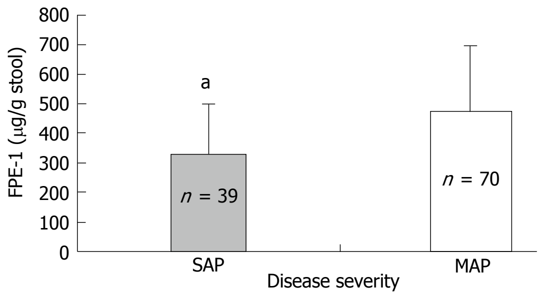

Figure 1 Disease severity and fecal pancreatic elastase-Ⅰ level. Fecal pancreatic elastase-Ⅰ (FPE-1) level was lower in severe acute pancreatitis (SAP) than mild acute pancreatitis (MAP) (^aP < 0.05). FPE levels are 330.9 ± 170.6 μg/g and 475.2 ± 223 μg/g stool in SAP and MAP, respectively.

stored at -20 ℃. FPE-1 level was measured by a commercially available Enzyme-Linked Immunosorbent Assay kit (Bioserv Diagnostics, BS-86-01, Rostock, Germany 2007) according to manufacturer's instructions. Stool elastase 1 concentration higher than 200 μg/g stool indicated normal pancreatic function, whereas concentration of 100 to 200 μg/g stool indicated mild to moderate pancreatic insufficiency, and concentrations below 100 μg/g stool were qualified as pointing to severe pancreatic insufficiency[12].

In all of the patients without diagnosed insulin-dependent diabetes mellitus (DM), endocrine pancreatic function was assessed by oral glucose tolerance test (OGTT). Measurement of glucose concentration was made on patients who had fasted overnight and discontinued drugs and food that could have affected the results. After taking blood samples for fasting blood glucose measurement during the OGTT, the patients drank 75 g glucose dissolved in 300 mL, and their blood glucose concentration was measured at 30, 60, 90 and 120 min. Basal blood glucose levels < 120 mg/dL and between 126-200 mg/dL at 120 min were accepted as impaired glucose tolerance. Basal glucose levels > 126 mg/dL and > 200 mg/dL at 120 min were accepted as DM.

The association between pancreatic dysfunction (either SCE or FPE-1 level) and disease severity and necrosis was investigated.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (Chicago, IL). Non-parametric tests were used to analyze the data. When comparing more than 3 groups, the Kruskal-Wallis test was used. Comparison between 2 groups was made with Mann-Whitney U test. The χ^2 test was used to compare categorical variables. A P value of < 0.05 was considered significant.

RESULTS

Patient demographics

A total of 109 patients with a mean follow-up of 32 mo (range: 6-48 mo) was included in this study. Fifty-eight of the subjects were male (53.2%), and 51 were female (46.8%). The mean age of the patients was 56.5 ± 15.7 (range: 19-89) years. The etiologies were biliary (66%), idiopathic (15.5%), alcohol (8.2%), hyperlipidemia (4.6%), endoscopic retrograde cholangio-pancreatography related (2.7%) and drug-related (2.7%). According to the APACHE Ⅱ scoring system, 35.8% of the patients were SAP, and the remaining were MAP. Necrosis was found in 27.5% of the patients, and there was only pancreatic edema in 49.6% of the patients on CECT examination. Almost 23% of the CECT findings were noted to be normal. While necrosis was found in 59% of the SAP cases, it was found in 10% of MAP. On the other hand, of the patients who had necrosis according to CECT findings, 76.6% had SAP, and the remaining had MAP according to APACHE Ⅱ criteria. Twenty-six percent of the patients who had pancreatic edema on CECT scan had SAP.

Five patients were operated on due to infected pancreatic necrosis, and a Beger procedure plus feeding jejunostomy was performed. Cystoenterostomy was performed in 17 cases due to a pancreatic pseudocyst. Percutaneous drainage was performed in 7 cases for pancreatic and peripancreatic abscesses. The remaining patients were treated medically. Before starting the study, 50.4% of the patients were taken pancreatic enzyme supplementation.

Exocrine dysfunction after acute pancreatitis

Exocrine dysfunction was detected in 13.7% of the patients according to both subjective clinical evaluation and the FPE-1 test. It was found in 17.9% with SAP and in 11.4% with MAP. Four patients in the SAP group had severe exocrine dysfunction according to FPE-1 measurement, and 11 patients had moderate exocrine dysfunction (7 of them in the MAP and 4 in the SAP group). Disease severity and necrosis were not associated with subjective clinical evaluation (Table 2). FPE-1 was lower in the SAP than MAP group and was lower in patients with necrotizing AP (NAP) than those without necrosis (Figures 1 and 2). FPE-1 was lower in cases with pancreatic head or near total necrosis than patients with necrosis at other localizations (Figure 3). This was under the critical level of FPE-1 (200 mg/g stool). There was no significant correlation between FPE-Ⅰ level and subjective clinical evaluation.

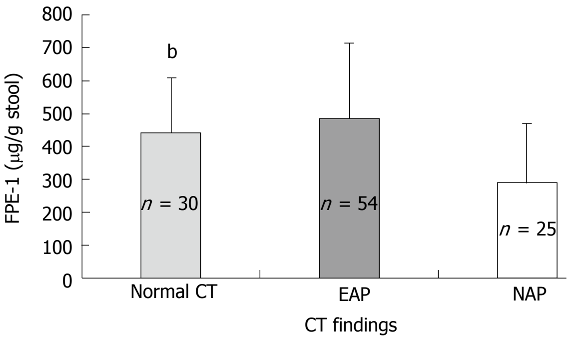

Figure 2 Fecal pancreatic elastase- I levels and contrast enhanced abdominal computed tomography findings. Fecal pancreatic elastase- I (FPE-1) level was significantly lower in necrotizing acute pancreatitis (NAP) than the patients without pancreatic necrosis (^{b}P < 0.001). FPE- I levels as follows; 292.43 ± 178.78, 487 ± 226.21, and 443.91 ± 167.83 μg/g stool in NAP, edematous acute pancreatitis (EAP) and normal contrast enhanced abdominal computed tomography (CT) groups, respectively.

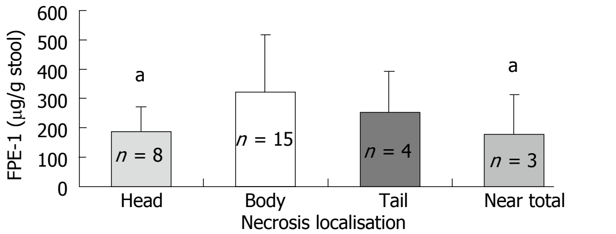

Figure 3 Necrosis localization and fecal pancreatic elastase- I levels. Fecal pancreatic elastase- I (FPE-1) level was lower in head and near total necrosis of the pancreas than body or tail necrosis (^{a}P < 0.05). FPE–1 levels as follows; 189.18 ± 82.7, 324.86 ± 194, 258.06 ± 134.63 and 181.12 ± 134.25 μg/g stool in head, body, tail and near total necrosis groups, respectively.

Table 2 The association between disease severity and contrast enhanced computed tomography findings and exocrine dysfunction *n* (%)

Disease severity and CT findings	Exocrine dysfunction	*P* value
SAP	7/39 (17.9)	NS
MAP	8/70 (11.4)	NS
NAP	8/30 (26.6)	
EAP	5/54 (9.2)	NS
Normal CT	2/25 (8.0)	

CT: Computed tomography; SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; NAP: Necrotizing acute pancreatitis; EAP: Edematous acute pancreatitis; NS: Not significant.

Endocrine dysfunction after acute pancreatitis

DM was detected in 11.9% of the cases before the AP attack. DM and impaired glucose tolerance were detected in 30.2% and 4.5% of the remaining cases, respectively, according to the OGTT test. Therefore, endocrine dysfunction was noted to be present in 34.7% of the cases (56.4% with SAP and 23.2% with MAP). According to CECT findings, patients with necrosis had more severe endocrine dysfunction than patients without necrosis (endocrine dysfunction rate was 66.6% in NAP, 27.8% in Edematous AP (EAP) and 12% in normal CECT) (Figure 4).

DISCUSSION

AP is a mediator disease caused by proinflammatory cytokine release and has a clinical picture ranging from mild disease to multiorgan failure and sepsis. The clinical picture is serious in 15%-20% of patients; complications can develop and mortality can be seen in these patients. Pancreatic necrosis develops in 20%-30% of the patients[3]. In this series, SAP was diagnosed in 35.8% and NAP was determined in 27.5% of the AP cases. The fact that SAP and NAP rates in our series are higher than those in the literature might be due to our hospital being the tertiary care referral center.

Currently, there is no consensus on whether or not pancreatic functions recover and to what extent after an AP attack. Full pancreatic functional recovery has been reported in some studies[6,19,20], whereas others have reported that both endocrine and exocrine insufficiency might develop[8-10]. The research is not conclusive or sufficient to answer the question. The results of previous studies remain conflicting because of very small patient numbers and non-homogenous etiologies that affect pancreatic functions. Using ineffective and non-standardized tests can also lead to some mistakes and controversies[11]. In the current study, we used subjective clinical evaluation and the FPE-1 test, which has a relatively high sensitivity for exocrine function. Pancreatic elastase is a specific protease of humans and it undergoes minimal breakdown during intestinal transit. Strong parallelism between stool FPE-1 and amylase, lipase and trypsin in pancreatic juice has been reported[21,22]. The sensitivity of the FPE- I test is 90% in SAP and 60%-70% in mild disease[23]. Although the test is easy and used widely in many reference laboratories, it is not an ideal test. But, FPE-1 test is more reliable than the direct tests which are more expensive, invasive and time-consuming.

Long term results after AP, in terms of pancreatic functions are heterogeneous. The pancreatic dysfunction rate after AP ranges between 11%-85% in SAP and 13%-55% in MAP[12,15,16,24]. Bozkurt *et al*[14] reported the results of their relatively small series. They observed mild-moderate exocrine dysfunction rates of 74% and 81% at 1 and 18 mo follow-up, respectively. Severe exocrine dysfunction following AP was noted in 26% at 1 mo and 6% at 18 mo. On the other hand, Ibars *et al*[11] observed normal pancreatic exocrine function after AP in their study of the same size. In our study, the exocrine dysfunction rate was found to be 13.7% among the patients. This rate was higher in the NAP group and lower in edematous cases and among patients with normal CECT findings. The duration between the AP attack and FPE-1 test of patients having exocrine dysfunction was relatively long (most of them longer than 24 mo). The relation between pancreatic exocrine dysfunction and pancreatic necrosis and its localization is not clear. Although no relation was found between necrosis and subjective clinical evalua-

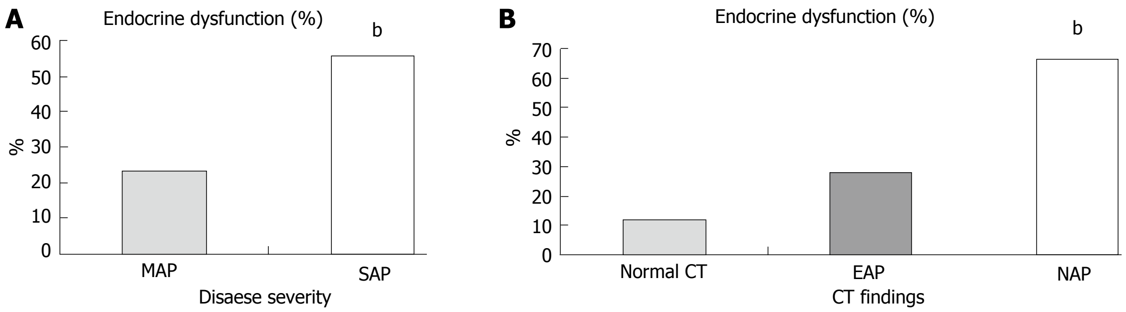

Figure 4 Acute pancreatitis and endocrine dysfunction. Endocrine dysfunction was much higher in severe acute pancreatitis than mild acute pancreatitis (A) and also much higher in necrotizing acute pancreatitis than without necrosis (B) (^bP < 0.001). SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; NAP: Necrotizing acute Pancreatitis; EAP: Edematous acute pancreatitis.

tion, the FPE-1 level was low in SAP, NAP and patients with pancreatic head necrosis. FPE-1 was noted to be under the critical threshold (< 200 μg/g stool) in patients having head necrosis. Kemppainen *et al*[25] reported that in patients with pancreatic head and body necrosis, the complication rate was much higher than the others and they attributed this to proximal obstruction.

As seen in some of our cases, the occurrence of exocrine dysfunction or enzyme insufficiency after AP may be independent of organ necrosis and severity of disease. We can only speculate about the cause of this finding. It is not ethical to biopsy the pancreas after AP. On the other hand, measuring tissue microcirculation and organ perfusion is not practical. We can speculate that fibrosis and the loss of functional units might occur during the healing period under cytokine cascade after AP attack. It has been reported that pancreatic exocrine dysfunction is relatively more common in the long term, especially after NAP, and in patients with necrosectomy[15,26]. Exocrine dysfunction was observed in 26.6% of NAP cases and 40% of patients who had necrosectomy in our study. We did not analyze the FPE-1 level in different time periods. Therefore, from our data, we cannot draw conclusions about the course of enzyme insufficiency over the long term.

We have very limited data about pancreatic enzyme supplementation and its dosage and duration after AP. Approximately 50% of our cases were on enzyme supplementation before starting the study. Interestingly, only 12.8% of these patients had exocrine dysfunction after one month tapering of enzyme supplementation. On the other hand, only 3 of 15 patients with exocrine dysfunction had been taking enzyme supplementation. Therefore these results showed that enzyme supplementation regimes should be questioned.

The reported rate of endocrine dysfunction after AP is 15%-35%[18,27]. The rate in the present study was relatively high (34.7%). In previous studies, endocrine dysfunction has been reported to occur in up to 50%-70% of cases of necrosis or necrosectomy[5,17,28]. In our study, endocrine dysfunction was related to disease severity and presence of necrosis but was not related to the necrosis ratio and localization. This finding can be also explained with pancreatic fibrosis occurring as a result of inflammation, leading to exocrine dysfunction. In addition to beta cell loss, increased insulin resistance is thought to be another causal factor for endocrine dysfunction after AP[17]. This factor was demonstrated in an experimental study[29]. In contrast to exocrine dysfunction, endocrine dysfunction was not related to pancreatic head necrosis. This also can be explained by the fact that islet cells are disseminated homogenously through the organ. Therefore islet cells localized in uninjured regions of the organ can compensate. Endocrine dysfunction can occur without necrosis if the whole pancreas is affected by fibrosis.

Necrosis is a risk factor for pancreatic dysfunction; however, pancreatic dysfunction can occur without necrosis over the long term. It is not easy to detect pancreatic dysfunction, especially exocrine dysfunction. Reasons for this include: (1) There is no ideal test to detect exocrine dysfunction; (2) Pancreatic dysfunction can be explained by apoptosis in patients who did not have necrosis; (3) Ultrastructural changes and fibrosis due to the healing process in the ductal system can cause dysfunction; and (4) Recurrent attacks might be a source of morphologic changes and dysfunction.

In conclusion, long-term quality of life after AP should be evaluated in SAP, NAP, patients having necrosectomy and patients with pancreatic head necrosis.

COMMENTS

Background

Eighty percent of the pancreatic mass is devoted to exocrine function, and the remaining part is responsible for endocrine function, which is crucial to the maintenance of homeostasis of the body. This study aimed to clarify the relationship between pancreatic dysfunction, the severity of the disease and the extent of the pancreatic necrosis.

Research frontiers

To evaluate the effects of disease severity and necrosis on organ dysfunctions in acute pancreatitis.

Innovations and breakthroughs

Necrosis is a risk factor for pancreatic dysfunction; however, pancreatic dysfunction can occur without necrosis over the long term. It is not easy to detect pancreatic dysfunction, especially exocrine dysfunction.

Peer review

In this study the authors evaluate the effects of disease severity and necrosis on organ dysfunction in acute pancreatitis. Fecal pancreatic Elastaz-1 and oral glucose tolerance test are used to measure exocrine and endocrine insufficiency. This is an interesting manuscript dealing with an area of investigation

poorly explored in the past.

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BRIEF ARTICLE

Shugan-decoction relieves visceral hyperalgesia and reduces TRPV1 and SP colon expression

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Abstract

AIM: To evaluate the therapeutic effect of Shugan-decoction (SGD) on visceral hyperalgesia and colon gene expressions using a rat model.

METHODS: Ninety-six adult male Wistar rats were randomized into six equal groups for assessment of SGD effects on psychological stress-induced changes using the classic water avoidance stress (WAS) test. Untreated model rats were exposed to chronic (1 h/d for 10 d consecutive) WAS conditions; experimental treatment model rats were administered with intragastric SGD at 1 h before WAS on consecutive days 4-10 (low-dose: 0.1 g/mL; mid-dose: 0.2 g/mL; high-dose: 0.4 g/mL); control treatment model rats were similarly administered with the irritable bowel syndrome drug, dicetel (0.0042 g/mL); untreated normal control rats received no drug and were not subjected to the WAS test. At the end of the 10-d WAS testing period, a semi-quantitative measurement of visceral sensitivity was made by assessing the abdominal withdrawal reflex (AWR) to colorectal balloon-induced distension (at 5 mmHg increments) to determine the pain pressure threshold (PPT, evidenced by pain behavior). Subsequently, the animals were sacrificed and colonic tissues collected for assessment of changes in expressions of proteins related to visceral hypersensitivity (transient receptor potential vanilloid 1, TRPV1) and sustained visceral hyperalgesia (substance P, SP) by immunohistochemistry and real-time polymerase chain reaction. Inter-group differences were assessed by paired t test or repeated measures analysis of variance.

RESULTS: The WAS test successfully induced visceral hypersensitivity, as evidenced by a significantly reduced AWR pressure in the untreated model group as compared to the untreated normal control group (190.4 ± 3.48 mmHg *vs* 224.0 ± 4.99 mmHg, $P < 0.001$). SGD treatments at mid-dose and high-dose and the dicetel treatment significantly increased the WAS-reduced PPT (212.5 ± 2.54, 216.5 ± 3.50 and 217.7 ± 2.83 mmHg respectively, all $P < 0.001$); however, the low-dose SGD treatment produced no significant effect on the WAS-reduced PPT (198.3 ± 1.78 mmHg, $P > 0.05$). These trends corresponded to the differential expressions observed for both TRPV1 protein (mid-dose: 1.64 ± 0.08 and high-dose: 1.69 ± 0.12 *vs* untreated model: 3.65 ± 0.32, $P < 0.001$) and mRNA (0.44 ± 0.16 and 0.15 ± 0.03 *vs* 1.39 ± 0.15, $P < 0.001$) and SP protein (0.99 ± 0.20 and 1.03 ± 0.23 *vs* 2.03 ± 0.12, $P < 0.01$) and mRNA (1.64 ± 0.19 and 1.32 ± 0.14 *vs* 2.60 ± 0.33, $P < 0.05$). These differential expressions of TRPV1 and SP related to mid- and high-dose SGD treatments were statistically similar to the changes induced by dicetel treatment. No signs of overt damage to the rat system were observed for any of the SGD dosages.

CONCLUSION: Shugan-decoction can reduce chronic stress-induced visceral hypersensitivity in rats, and the regulatory mechanism may involve mediating the expressions of TRPV1 and SP in colon tissues.

Key words: Shugan-decoction; Visceral hypersensitivity; Sustained visceral hyperalgesia; Water avoidance stress; Transient receptor potential vanilloid 1; Substance P

Core tip: The classical rat model of chronic stress induction *via* water avoidance stress (WAS) test was used to investigate the therapeutic effect of the Shugan-decoction (SGD) on visceral hypersensitivity of the gastrointestinal tract and its underlying molecular mechanisms. The study design reflected the therapeutic potential of SGD for treating the stress-related gut aspects of irritable bowel syndrome (IBS) in humans. Mid- and high-dose SGD treatments significantly increased the WAS-reduced pressure thresholds, similarly to those induced by the IBS drug dicetel. The SGD treatments also restored WAS-related changes in transient receptor potential vanilloid 1 and substance P expression in the colon.

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INTRODUCTION

In recent decades, irritable bowel syndrome (IBS) has emerged as a highly prevalent functional gastrointestinal disorder that is strongly associated with high levels of stress in daily life. The spectrum of IBS symptoms, ranging from discomfort associated with altered bowel habits to recurrent abdominal pain, is non-life threatening, but can severely impact an individual's general wellbeing and severely disrupt daily life. Despite the extensive laboratory- and clinical-based investigations that have been carried out to determine the underlying etiology and pathogenesis of IBS, no precise causative factors have been identified for the onset and progression of this disease. Patients present with an absence of IBS-specific structural and biochemical abnormalities[1,2], but have higher incidences of psychological stress (both acute and chronic), visceral sensory abnormalities, gastrointestinal motility disorders, and gastrointestinal infections.

The theory of increased visceral sensitivity as a feature of IBS has been addressed by numerous studies. Indeed, IBS patients have been reported to show an enhanced sensitivity to colon and rectal balloon dilatation[3]. The mechanisms underlying such visceral hypersensitivity remain unknown, but are likely multifactorial and complex[4,5]. Under normal physiological conditions, visceral sensitivity is mediated by a variety of neuron-localized ion channels, such as the transient receptor potential (TRP) non-selective cation channels, that also function in the formation and regulation of hyperalgesia.

The transient receptor potential vanilloid 1 (TRPV1) TRP family member plays a key role in modulation of the sensation of pain and thermal hyperalgesia[6] and is widely expressed throughout the gastrointestinal tract[7]. In the colon, substance P (SP)-mediated phosphorylation activates TRPV1, thereby enhancing the probability of channel gating promoting development of visceral hypersensitivity[8]. In this manner, SP itself acts as an important regulator of sustained visceral hyperalgesia, and has been characterized as an etiological factor of the repeated stress rat model system[9].

Clinical observations of IBS patients have indicated that remarkably aggravated disease symptoms occur during times of increased emotional and mental stress[10]. In traditional Chinese medicine (TCM), these disrupted states correspond to liver-depression and spleen-deficiency. Thus, therapies that soothe the liver and strengthen the spleen are applied to IBS patients. One such therapy is the TCM compound Shugan-decoction (SGD), which, when administered orally, has been shown to significantly improve the clinical symptoms of IBS[11]. In this study, a rat model of stress-induced visceral hypersensitivity was employed to investigate the efficacy profile and therapeutic mechanism of SGD in IBS-like conditions.

MATERIALS AND METHODS

Animals

Ninety-six Wistar rats (150 ± 20 g adult males) were obtained from the Experimental Animal Center of Shanghai University of TCM (China) for analysis. The animals were housed under a 12/12 light cycle, with standard temperature (21-23 ℃) and humidity (50% ± 5%) and *ad libitum* access to standard rat chow and tap water. All consecutive daily experimental procedures were conducted between 8:00-11:00 AM to minimize confounding due to diurnal variations.

The study was designed according to the guidelines of ethical treatment in research published by the Committee of International Association for the Study of Pain and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research at the Shanghai University of TCM. All protocols were carried out with the aim of minimizing or eliminating discomfort to the animals.

Experimental compounds

The constituent ingredients of SGD (white atractylodes rhizome, white peony root, dried old orange peel, Ledebouriella root and *Radix bupleuri*) were purchased as crude herbs from the Yanghetang Pharmacy (Shanghai, China). The aqueous extract of SGD was made by the Herbal Chemistry Lab at the Shanghai University of TCM, using

the following steps: decoction of the crude herbs twice, combination of the two filtrate samples, decompression recovery to obtain the final aqueous extract product. The standard IBS pharmaceutical drug dicetel (pinaverium bromide; 50 mg tablets) was obtained from Solvay Pharma (Suresnes, France).

Water avoidance stress test

Repeated water avoidance stress (WAS) was conducted as previously described to induce chronic psychological stress with gastric disruption[12]. Briefly, rats were placed on a clear glass platform (10 × 8 × 8 cm) in the middle of a plexiglass tank (45 × 25 × 25 cm) filled with water at 25 ℃ (to fill the tank up to 1 cm below the top of the platform), and remained on the platform for 1 h. The WAS procedure was repeated once daily for 10 consecutive days.

Treatment and control groups

Untreated model rats (n = 16) were exposed to chronic (1 h/d for 10 d consecutive) WAS conditions. Experimental treatment model rats (n = 16 each dosage group) were administered with intragastric SGD at 1 h before WAS on consecutive days 4-10 (low-dose: 0.1 g/mL; mid-dose: 0.2 g/mL; high-dose: 0.4 g/mL). Control treatment model rats (n = 16) were similarly administered the IBS drug, dicetel (0.0042 g/mL). Untreated normal control rats (n = 16) received no drug and were not subjected to the WAS test.

Measurement of fecal pellet output

To estimate distal colonic motility, fecal pellet output was measured as previously described[9]. Briefly, fecal pellets found in the WAS tank were counted at the end of each 1 h WAS test. For the untreated normal control rats, the amount of fecal pellets left in the home cage were counted over a 60 min period of time. Data are presented as mean ± SE (n = 16).

Colorectal distension and semi-quantitative measurement of pressure pain threshold

At the end of the 10-d WAS testing period, a semi-quantitative measurement of visceral sensitivity was made in each group (n = 8 each group) by assessing the abdominal withdrawal reflex to colorectal balloon-induced distension to determine the pressure threshold (evidenced by pain behavior)[10]. Briefly, rats were lightly sedated with halothane and a deflated latex balloon (4-5 cm diameter at full inflation) was inserted intra-anally with its end 1 cm proximal to the anus into the descending colon and rectum. Animals were then placed into a small lucite cubicle (20 × 8 × 8 cm) and allowed to wake up and adapt for 30 min prior to initiation of colorectal distension (CRD). The CRD was performed by progressive inflation of the colorectally-inserted balloon at 5 mmHg increments, and stopped when the animal exhibited pain behavior. The pressure pain threshold (PPT) value was recorded as the mmHg pressure that evoked contraction of the animal's abdominal muscles following balloon-mediated CRD delivered for 30 s duration at 4 min intervals. All the measurements were observed by two investigators (Shi HL and Qian W) working independently and blinded to the animals' grouping.

Sacrifice and colon tissue collection

All rats were sacrificed by cervical dislocation immediately after visceral sensitivity measurements were completed so that the descending colon (2 cm above the anus, which had not undergone CRD) could be removed by dissection. The tissue sample was then divided into two parts: one was fixed with 10% formalin [for subsequent immunohistochemical (IHC) analysis] and the other was snap-frozen and stored at -80 ℃ [for subsequent real-time polymerase chain reaction (PCR) analysis].

IHC analysis

The IHC analysis of TRPV1 and SP protein expression in colon tissues was performed using the EnVision + System two-step horseradish peroxidase staining technique (DakoCytomation, Glostrup, Denmark) with targeted polyclonal rabbit anti-human primary antibodies (1:100 dilutions; Santa Cruz Biotechnology, Inc., Santa Cruz, CA,United States). Negative controls were run with the primary antibodies omitted from the procedure. Positive detection was indicated by visualization of a brown stain in the cytoplasm. Three randomly selected × 200 magnification fields were evaluated using a BH2 microscope (Olympus, Tokyo, Japan) equipped with a Nikon 4500 digital camera (Tokyo, Japan). The computer-aided image analysis system by Qiu Wei Inc. (Shanghai, China) assessed the area and optical density (OD) of TRPV1 and SP-positive cells in each field. The IHC index was calculated as the average integral optical density: [(positive area × OD)/total area]. Data are presented as mean ± SE (n = 6).

Real-time PCR

Total RNA was extracted from the thawed colon tissue samples using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, United States) and reverse transcribed to cDNA by using the Prime-Script™ Reagent Kit (Takara, Tokyo, Japan), according to the manufacturers' instructions. The following primer sets (forward and reverse, respectively) were used for gene-specific amplifications: TRPV1 (GenBank accession No. NM_031982): 5'-CCACACAAGTGCCGGGGGTC-3' and 5'-CCAGGTCGCCCATGCCGATG-3'; SP (GenBank accession No. NM_053844): 5'-CTTCCTGGACGCGATGGGCTG-3' and 5'-TGGAAATCCTGGCAGGCCCCTT-3'; GAPDH (normalizing control; primers were synthesized by Dawei Biotechnology Co., Shijiazhuang, China): 5'-GCCACAGCACTCCATCGAC-3' and 5'-GTCTCCGATCTGGAAAACGC-3'. The real-time PCR was carried out with Synergy Brands Green I dye (Qiagen GmbH, Hilden, Germany) using a Prism 7500 System (Applied Biosystems Inc., Foster City, CA, United States) under the following conditions: 40 cycles of 94 ℃ for 30 s, 57 ℃ for 30 s and 72 ℃ for 30 s, followed by a single final extension cycle of 72 ℃ for 7 min.

Samples were run in triplicate and the normalized values were averaged. Data are presented as mean ± SE (n = 6).

Statistical analysis

All statistical analyses were carried out with the GraphPad Prism v5.0 software (GraphPad Software Inc, La Jolla, CA, United States). Inter-group differences were assessed by a paired t test or repeated measures analysis of variance. A P value of < 0.05 was set as the threshold for statistical significance.

RESULTS

WAS-induced visceral hypersensitivity and hyperalgesia are relieved by SGD

The WAS test successfully induced visceral hypersensitivity, as evidenced by a sustained significant increase in fecal pellet output (distal colonic motility) from the untreated model group as compared with the untreated normal control group not subject to the WAS test (day 3: 8.69 ± 0.60 *vs* 2.31 ± 0.66 and day 10: 8.56 ± 0.63 *vs* 0.56 ± 0.29, both P < 0.001). After 7 d of SGD treatment, significant relief of the WAS-stimulated increase in fecal output was achieved by the mid-dose (day 3: 8.38 ± 0.77 *vs* day 10: 4.31 ± 0.42, P < 0.001) and high-dose (day 3: 8.19 ± 0.62 *vs* day 10: 3.63 ± 0.39, P < 0.001). Although the extent of relief in these groups was similar to that achieved with the dicetel control treatment (day 3: 8.75 ± 0.53 *vs* day 10: 4.00 ± 0.35, P < 0.001 for all *vs* corresponding mid- and high-dose SGD values), none of the treatments reduced fecal output to untreated normal control group levels by day 10. The low-dose SGD treatment produced no significant effect on WAS-stimulated fecal output increase (day 3: 8.94 ± 0.84 and day 10: 6.88 ± 0.51; both P < 0.001 *vs* untreated normal control group; P > 0.05 for day 3 *vs* day 10).

The same WAS-induced and SGD-relieved trends were seen for visceral hyperalgesia. The untreated model group showed significantly lower PPT than the untreated normal control group (190.40 ± 3.48 mmHg *vs* 224.00 ± 4.99 mmHg, P < 0.001), which was relieved by the mid- and high-dose SGD treatments (212.50 ± 2.54 mmHg and 216.50 ± 3.50 mmHg) to a similar extent achieved with dicetel control treatment (217.70 ± 2.83 mmHg) (all P < 0.001 *vs* untreated model group). Again, the low-dose SGD treatment produced no significant effect on the WAS-reduced pressure threshold (198.30 ± 1.78 mmHg, P > 0.05 *vs* untreated normal control group and P < 0.001 *vs* untreated model group).

WAS-reduced expression of colon-expressed genes related to visceral hypersensitivity (TRPV1) and hyperalgesia (SP) was relieved by SGD treatment

IHC detection of TRPV1 and SP in colon tissues of untreated normal control rats showed that their expressions were mainly localized to the mucosa and submucosa (Figure 1). The untreated model group showed significantly higher AOID levels than the untreated normal controls for both TRPV1 (3.65 ± 0.32 *vs* 0.86 ± 0.11, P < 0.001) and SP (2.03 ± 0.12 *vs* 0.64 ± 0.11, P < 0.001). These WAS-stimulated increases in protein levels were significantly reduced by the SGD treatments at mid-dose (TRPV1: 1.64 ± 0.08 and SP: 0.99 ± 0.20) and high-dose (TRPV1: 1.69 ± 0.12 and SP: 1.03 ± 0.23) compared with the untreated model group (TRPV1: P < 0.001 and SP: P < 0.01). Furthermore, the extent of reduction was similar to that achieved with dicetel control treatment (TRPV1: 1.46 ± 1.60 and SP: 0.76 ± 0.11; both P < 0.001 *vs* untreated model group). The low-dose SGD treatment produced no significant effect on the WAS-stimulated increases in TRPV1 (3.48 ± 0.33, P < 0.001 *vs* untreated model group) or SP (1.69 ± 0.22, P < 0.01 *vs* untreated model group).

The same WAS-induced and SGD-relieved trends were seen for the gene expressions of TRPV1 and SP. The untreated model group showed significantly higher relative expressions of both genes compared with the untreated normal control group (TRPV1: 1.39 ± 0.15 *vs* 0.14 ± 0.03 and SP: 2.60 ± 0.33 *vs* 0.70 ± 0.12, both P < 0.001). The mid- and high-dose SGD treatments significantly reduced the WAS-increased mRNA expression of TRPV1 (0.44 ± 0.16 and 0.15 ± 0.03, both P < 0.001 *vs* untreated model group) and SP (1.64 ± 0.19 and 1.32 ± 0.14, both P < 0.05 *vs* untreated model group), with the SP levels being uniquely reduced by mid-dose SGD to levels similar to those of the untreated normal controls (P < 0.05). Furthermore, the trends in SGD-mediated relief were similar to those observed with the dicetel control treatment (TRPV1: 0.22 ± 0.02, P < 0.001 *vs* untreated model group and SP: 1.35 ± 0.13, P < 0.01 *vs* untreated model group). Again, the low-dose SGD treatment produced no significant effect on the WAS-stimulated increase in mRNA expression of TRPV1 (0.99 ± 0.16) and SP (2.34 ± 0.19) (both P < 0.001 *vs* untreated normal control group).

DISCUSSION

In the present study, the well-established animal model of chronic water avoidance stress was used to stimulate the gastrointestinal tract hypersensitivity that is characteristic of human IBS. The WAS-induced physical manifestations (*i.e.*, increased fecal output and lower PPT) were accompanied by differential expression patterns of genes/proteins related to visceral hypersensitivity (TRPV1) and hyperalgesia (SP) in colon tissues. In addition, the model was used to evaluate the therapeutic efficacy of SGD, as a TCM alternative to dicetel, the pharmacologic agent most commonly used to treat IBS in humans. The findings indicated SGD was able to relieve the WAS-induced visceral hypersensitivity and hyperalgesia, as well as restore the perturbed TRPV1 and SP expressions.

Visceral pain, related to CRD and visceral hypersensitivity, is a hallmark feature of IBS and is often the factor precipitating a patient's presentation to the clinic[13-16]. However, the underlying molecular mechanisms of the

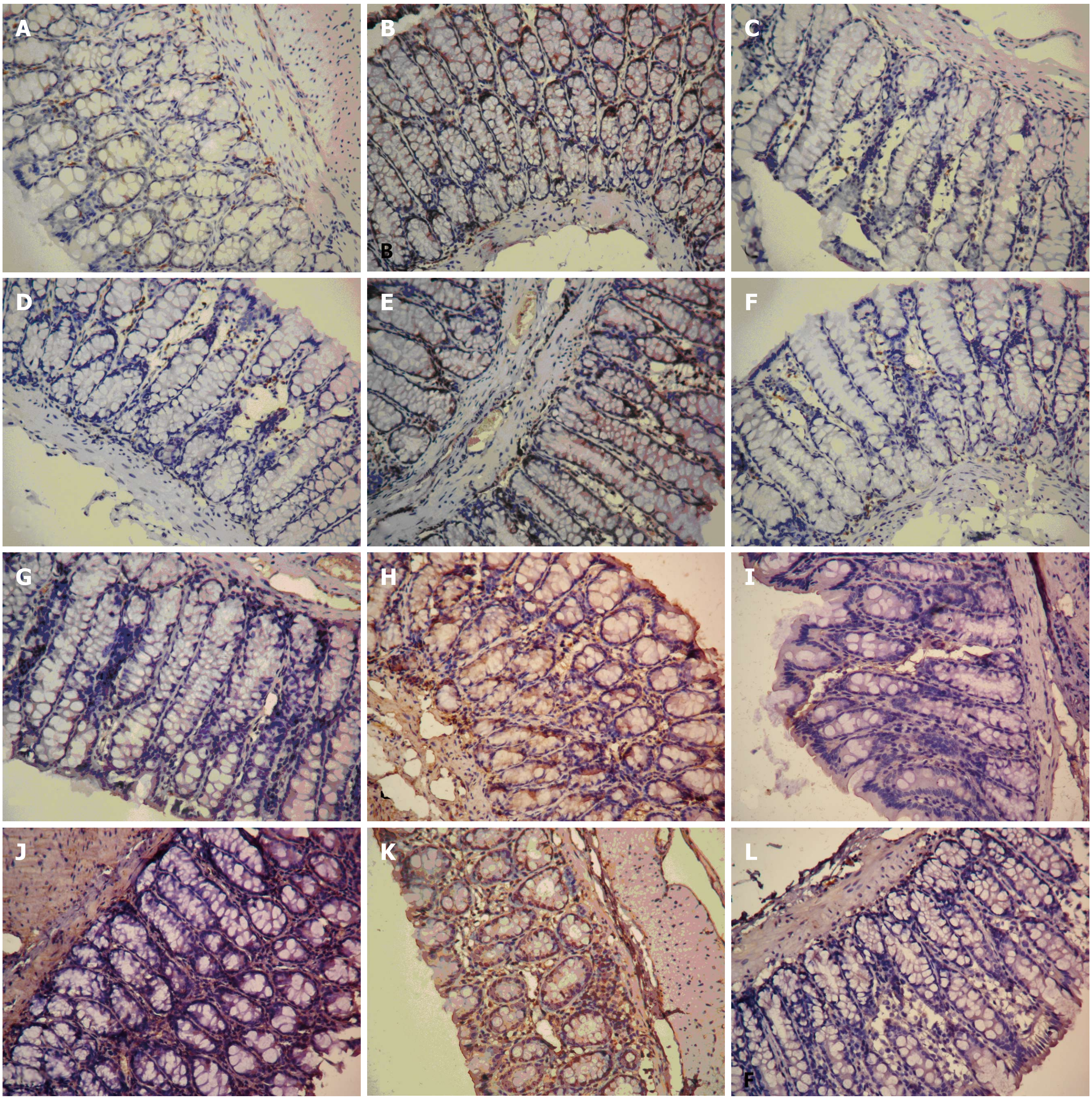

Figure 1 Reductions in the expressions of substance P and transient receptor potential vanilloid 1 protein by water avoidance stress in the colon are relieved by SGJGD treatment. A-F: Substance P protein; G-L: Transient receptor potential vanilloid 1. IHC-detected colon tissues (× 200) from: untreated normal group (A, G); untreated model group (B, H); high-dose Shugan-decoction (SGD) model group (C, I); mid-dose SGD model group (D, J); low-dose SGD model group (E, K); dicetel control model group (F, L).

IBS pain response are poorly understood, which has inhibited development of effective pain management strategies[17,18]. The demonstration of TRPV1 as a contributor to WAS-induced colonic hypersensitivity, suggests its potential as a target of molecular therapies that may not only reduce the overactive distal colonic motility, but also relieve the associated lower PPT. Indeed, when TRPV1 was knocked-out in mice, the visceral sensitivity to CRD was significantly reduced[19], and enhanced TRPV1 expression has been observed in a variety of gastrointestinal diseases[20,21], including human cases of IBS[22].

An increased amount of TRPV1-expressing nerve fibers have been reported in IBS-affected tissues from human patients[22], and may represent a physiological link between increased TRPV1 transcription and the pain response in IBS[23,24]. In addition, inflammatory factors are known agonists of TRPV1 channels[25] and might explain the common feature of low-grade inflammation in IBS. Considering a previous finding that development of fecal urgency and rectal hypersensitivity correlated with increased immunoreactivity to TRPV1 within the gastrointestinal tract[20], it is possible that therapeutic antagonism of TRPV1 channels may result in antihyperalgesic effects without hypoalgesic activity, and might be beneficial in the treatment of IBS visceral pain[26].

The current study's finding of chronic WAS-induced

changes in SP colon expression agree with other recent studies using the same model system that have implicated this neuropeptide in the maintenance of visceral hyperalgesia[9,27]. As a critical neurotransmitter of injurious signals, SP effectively links the gut nervous system to the immune system, stimulating a wide range of effector cells in the stomach and intestine to facilitate proper gastrointestinal motility, sensibility, secretion and absorption. The mechanism by which SP mediates visceral hypersensitivity may involve a myriad of cellular processes and signaling cascades, including promotion of the mast cell degranulation response, the release of histamines, leukotrienes, prostaglandins and bradykinin, all of which can cause inflammatory reactions leading to neuropathic pain[28].

The clinical observations of increased SP expression in the intestinal mucosa of IBS patients[29,30], coupled with the previous demonstration of SP's ability to activate TRPV1 *via* phosphorylation, thereby enhancing the probability of channel gating[9], suggested that SP might be a vital mediator of chronic stress-induced visceral hyperalgesia through the modulation of TRPV1 channels. When TRPV1 channels are activated, a large Ca^{2+} influx can lead to cellular depolarization[31], with neurons releasing an array of neurotransmitters to trigger the downstream response of visceral hypersensitivity.

Dicetel is the most commonly applied pharmacotherapy of IBS, yet it is associated with a wide range of side effects, such as itching, rash, nausea and dry mouth. In addition, its widespread adoption in clinical practices worldwide has been hampered by its high monetary cost. In the current study, SGD treatment led to decreased expression of the WAS-stimulated TRPV1 and SP proteins and mRNAs in the hypersensitive colon, and increased the pain threshold of the rats. Thus, SGD appears to be an effective alternative to the pharmacologic agent dicetel for treating IBS by affecting the transcription and translation (and presumably secretion) of TRPV1 and SP in the colon.

In conclusion, the TCM SGD is an effective agent for reducing WAS-induced expressions of TRPV1 and SP in rat colons, thereby reducing visceral hypersensitivity and hyperalgesia. However, the chronic WAS testing (10 consecutive days) used in this study caused no overt damage to the colon's histological structure (data not shown), which may be a limitation in the study's findings, because human IBS is accompanied by significant structural changes (likely associated with the inflammatory component of IBS). Nonetheless, the present findings indicate an underlying mechanism of stress-induced disruption of distal colon motility and pain, which may represent useful targets for molecular based therapies to treat the pain and sensitivity symptoms of abdominal diseases, such as IBS.

COMMENTS

Background

Visceral hypersensitivity has been proposed as a significant contributor to the pathophysiology of irritable bowel syndrome (IBS). Activation of the transient receptor potential vanilloid 1 (TRPV1) channel on neurons, by such effector molecules as the neurotransmitter substance P (SP), increases the probability of channel gating and promotes the formation of visceral hypersensitivity. Therefore, SP-mediated activation of TRPV1 might play a role in the visceral hypersensitivity and hyperalgesia induced by chronic stress conditions, as in IBS. The traditional Chinese medicine (TCM) compound Shugan-decoction (SGD) has been shown to significantly improve the clinical symptoms of IBS patients; however, the therapeutic mechanism of SGD remains unknown.

Research frontiers

The molecular mechanisms underlying IBS remain to be fully elucidated, and may represent useful targets of therapies to relieve not only the symptoms associated with visceral hypersensitivity (increased distal colonic motility), but also those related to visceral hyperalgesia (abdominal pain, possibly related to an overactive inflammatory response). In this study, the classical rat model of chronic stress inducement *via* the water avoidance stress (WAS) test was used to investigate the underlying molecular mechanisms of visceral hypersensitivity and hyperalgesia in the gastrointestinal tract and to evaluate the related therapeutic effect of SGD for treating the stress-related gut aspects of IBS in humans. Mid- and high-dose SGD treatments significantly increased the WAS-reduced pressure thresholds and restored WAS-related changes in TRPV-1 and SP expression in the colon, suggesting this TCM compound as a feasible alternative to the pharmacological agent dicetel.

Innovations and breakthroughs

This study provided novel insights into the molecular mechanisms underlying the observations of SGD-mediated improvements in the clinical symptoms of IBS. Specifically, SGD was demonstrated to reduce WAS-induced perturbations in TRPV1 and SP expressions in the colon that accompany visceral hypersensitivity and hyperalgesia.

Applications

The finding that SGD may reduce WAS-induced visceral hypersensitivity and hyperalgesia through regulation of the colonic expressions of TRPV1 and SP confirm this TCM compound as a useful prescription for the treatment of abdominal pain in IBS.

Terminology

Shugan-decoction is made according to the classic Tongxieyaofang recipe and is reported to soothe the liver soothing and strengthen the spleen. Irritable bowel syndrome is a functional gastrointestinal disorder that is associated with high levels of stress in daily life, and manifests as altered bowel habits and recurrent abdominal pain. The water avoidance stress test is a well-established technique for inducing chronic psychological stress with gastric disruption in a rat model system. TRPV1 is widely expressed on neurons throughout the gastrointestinal tract and modulates visceral sensitivity and hyperalgesia. SP is a neurotransmitter that activates TRPV1 and regulates visceral hyperalgesia.

Peer review

The authors investigated the therapeutic potential and underlying molecular mechanisms of the TCM compound SGD, in comparison to the common IBS pharmacologic agent dicetel, to relieve stress-induced visceral hypersensitivity and hyperalgesia. This is an interesting manuscript, and the general design is acceptable.

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BRIEF ARTICLE

Clinical characteristics and prognosis of young patients with colorectal cancer in Eastern China

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Abstract

AIM: To explore the clinical characteristics and prognosis of young patients with colorectal cancer patients in Eastern China.

METHODS: A total of 1335 patients with colorectal cancer treated from December 1985 to December 2005 at the Second Affiliated Hospital of Zhejiang University School of Medicine were studied retrospectively. The patients were divided into two groups, a younger group (aged ≤ 30 years) and an older group (aged > 30 years), and comparison was made in the clinical characteristics and prognosis between the two groups. Chi-square test was used for data analysis of all categorical variables, and overall survival (OS) was calculated by the Kaplan-Meier method. A multivariate analysis was performed using the Cox model.

RESULTS: There were 42 (3.1%) and 1293 (96.9%) cases in the younger group and older group, respectively. Univariate analysis showed that the 5- and 10-year OS in the younger group were 33.9% and 26.1%, respectively, and those in the older group were 60.1% and 52.2%, respectively. Younger group had poor survival (χ^2 = 14.146, P = 0.000). Multivariate analysis revealed that age was not a dependent factor for prognosis (OR = 0.866, 95%CI: 0.592-1.269, P = 0.461). Stratified analysis indicated that in stage Ⅲ and Ⅳ disease, the 5- and 10-year OS were 24.6% and 14.8% in the younger group, and 40.4% and 33.3% in the older group, respectively, with a significant difference between the two groups (χ^2 = 5.101, P = 0.024). In the subgroup of radical surgery, the 5- and 10-year OS were 44.3% and 34.2% in the younger group, and 69.6% and 60.5% in the older group, with a difference being significant between the two groups (χ^2 = 7.830, P = 0.005).

CONCLUSION: Compared with older patients, the younger patients have lower survival, especially in the subgroups of stage Ⅲ and Ⅳ disease and radical surgery.

Key words: Colorectal cancer; Young; Clinicopathologic feature; Prognosis; Radical surgery

Core tip: We firstly described the clinical characteristics and prognosis of young patients with colorectal cancer in Eastern China. The incidence rate of colorectal

cancer in young patients was higher than that in other reports. Younger patients with colorectal cancer had more poorly differentiated and advanced tumors, and worse prognosis, especially patients with stage Ⅲ and Ⅳ disease.

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INTRODUCTION

As a kind of common cancer, colorectal cancer severely threatens the health of people. Colorectal cancer is the fourth common cancer and the second leading cause of cancer death in the world[1]. The majority of patients are affected in their 50s to 70s, but the age at diagnosis is getting younger[2]. The annual percentage of colorectal cancer in young people is increasing[2]. There has been an increasing number of reports about young colorectal cancer patients in recent years. The outcomes of young colorectal cancer patients varied widely among different regions[2-4]. The incidence rate of colorectal cancer in young patients has also been increasing in recent years in China[5]. Nearly all reports showed that young colorectal cancer patients had specific clinicopathologic characteristics, including poor histological feature, and more mucinous tumors, signet ring cell tumors, and advanced tumors[6-8]. However, the relationship between the age and survival was not confirmed. Some reports documented that young colorectal cancer patients had worse survival compared with the older counterparts[2,7-9]. But the others indicated opposite results[10-15]. There are still controversies about the definition of the age of young population. This study was to retrospectively analyze the data of patients with colorectal cancer who received surgery at our center over the past 30 years. Based on the distribution of the age, the population with colorectal cancer aged < 30 years was considered as a special subgroup in our center. Therefore, the young population was defined as those aged ≤ 30 years in our study. This study was designed to explore the clinicopathologic characteristics and prognosis of young colorectal cancer patients in Eastern China.

MATERIALS AND METHODS

A total of 1335 consecutive patients with colorectal cancer (aged 19-92 years, mean 58 ± 13.3 years) treated from December 1985 to December 2005 at the Second Affiliated Hospital of Zhejiang University School of Medicine, located in Eastern China, were studied retrospectively. The patients were divided into two groups, a younger group (42 cases, aged ≤ 30 years, average age, 26.0 ± 3.5 years) and an older group (1293 cases, aged > 30 years, average age 58.0 ± 12.3 years). The criteria for inclusion were as follows: (1) patients with pathologically confirmed colorectal cancer; and (2) patients who underwent operations, including palliative surgeries. Patients with anal cancer or non-adenomas were excluded. Following the approval by the ethics committee of the hospital, the data including age, gender, tumor location, histological grade, approach of surgery, tumor infiltration, number of metastatic lymph nodes, distant metastasis and survival were obtained. Follow-up was made every 3 mo for 2 years, 6 mo for 5 years, then every one year. The follow-up proceeded through telephone calls or mail correspondence. The events of relapse and death in all patients were recorded.

The deadline of follow-up was November 2011. The follow-up lasted 0-302 mo (median, 57.0 ± 68.1 mo). Finally, 1335 patients who had complete data were analyzed; 267 patients (20.0%) were lost to follow-up, with 5 patients (11.9%) in the younger group and 262 patients (20.3%) in the older group. There was no significant difference in the percentage of lost patients between two groups (P = 0.183). The lost patients were taken as censors when the survival was analyzed. Twenty-nine patients died of colorectal cancer in the younger group and 604 patients died in the older group, including 51 patients who died due to other causes. They were considered as censors when cancer-related survival was calculated. All 1335 cases were included when we analyzed the clinicopathologic difference between the two groups.

The tumor was staged according to the 7th pathologic TNM staging system of AJCC[16]. Tumor location was described in detail as the cecum, ascending colon, liver flexure colon, transverse colon, descending colon, sigmoid, sigmorectal junction and rectum. Overall survival was calculated from the time of operation to death. Cancer-related survival was from the time of operation to the date of death because of the colorectal cancer. Causes of non-special cancer-related death included benign disease, accident, and secondary cancer. Radical surgery was classified as a procedure for no residual tumor left behind microscopically at resection margins. Palliative surgery was defined as a procedure for the residual tumor left macroscopically, which also included bypass or ileostomy. All palliative surgeries were considered as non-radical surgery.

Statistical analysis

Data of all categorical variables are summarized using frequencies and percentages. The data were analyzed with χ^2 test. Overall survival was calculated according to the Kaplan-Meier method. Survival rates were compared by the log-rank test. A multivariate analysis was performed using the Cox model. When a P-value was less than 0.05, the difference was considered significant. SPSS 16.0 statistical software was used for data analysis.

RESULTS

Clinicopathologic characteristics

The patient age ranged from 19 to 92 years, with a median of 58 ± 13.3 years. There were 42 (3.1%) and 1293 (96.9%) cases in the younger group and older group, respectively. The ratio of male to female was 1.3:1 in both groups.

The rectum was the frequent location in colorectal cancer, with a slightly higher rate in the younger group than in the older group (59.5% *vs* 49.3%, $P > 0.05$). Compared with the older group, significantly more patients in the younger group had mucinous tumor (33.3% *vs* 13.8%, $P = 0.000$), signet ring cell cancer (7.1% *vs*1.7%, $P = 0.010$) and poorly differentiated tumor (59.5% *vs* 15.7%, $P = 0.000$).

As for tumor infiltration, no tumor *in situ* (Tis) was found in the younger group, but 17 (13.1%) patients in older group were diagnosed with tumor *in situ* (Tis). Interestingly, there was no significant difference between the two groups in the tumor infiltration ($P = 0.264$). The percentages of patients with lymph node metastasis (≥ 4 lymph nodes), distance metastasis, stage Ⅳ and stage Ⅰ disease and radical surgery were 35.7%, 28.6%, 31.0%, 2.4% and 66.7%, respectively, in the younger group, and 14.2%, 15.4%, 15.5%, 30.2% and 83.7%, respectively, in the older group, with significant differences between the two groups ($P = 0.021$, 0.021, 0.007, 0.008 and 0.008, respectively) (Table 1).

Overall survival

Univariate analysis showed that there was a significant difference in total overall survival between the two groups ($\chi^2 = 14.146$, $P = 0.000$) (Figure 1, Table 2). Multivariate analysis revealed that age was not an independent factor for the prognosis of colorectal cancer (OR = 0.866, 95%CI: 0.592-1.269, $P = 0.461$). TNM stage Ⅲ/Ⅳ, the approach of palliative surgery, rectal cancer, mucinous cancer and poorly differentiated tumor were independent factors for worse prognosis (Table 2).

As for stage Ⅰ and Ⅱ disease, the 10-year overall survival and median survival time had not reached until the deadline in the younger group, which might be due to the small sample size of the study. There was no significant difference in the 10-year overall survival and median survival time between the two groups ($\chi^2 = 0.016$, $P = 0.899$) (Figure 2A, Table 3). Fifty-one patients died of other diseases in the older group. In order to diminish the influence of the non-cancer death, the cases in the subgroup of stage Ⅰ and Ⅱ disease were analyzed; as a result, there was also no difference in cancer-related survival between the two groups ($\chi^2 = 0.356$, $P = 0.551$) (Figure 2B). For stage Ⅲ and Ⅳ disease, the outcome was worse in the younger group than in the older group ($\chi^2 = 5.101$, $P = 0.024$) (Figure 3, Table 3). For the subgroup of radical surgery, in the older group, the median survival time had not reached until the deadline. There was a significant difference in median survival time between the two groups ($\chi^2 = 7.830$, $P = 0.005$) (Figure 4A, Table 3). In the non-radical surgery subgroup, there was no significant difference in median survival time between the two groups ($\chi^2 = 0.112$, $P = 0.737$) (Figure 4B, Table 3).

Table 1 Clinical and pathologic characteristics of colorectal cancer in the younger group and older group *n* (%)

Variable	Younger group (n = 42) (≤ 30 yr)	Older group (n = 1293) (> 30 yr)	*P* value
Gender			NS
Male	24 (57.1)	738 (57.1)	
Female	18 (42.9)	555 (42.9)	
Location of tumor			
Cecum	1 (2.4)	69 (5.3)	
Ascending colon	1 (2.4)	154 (11.9)	
Hepatic flexure	1 (2.4)	86 (6.7)	
Transverse colon	2 (4.8)	52 (4.0)	
Splenic flexure	1 (2.4)	29 (2.2)	
Descending colon	6 (14.3)	49 (3.8)	
Sigmoid	5 (11.9)	201 (15.5)	
Rectosigmoid junction	0 (0)	16 (1.2)	
Rectum	25 (59.5)	637 (49.3)	0.191[1]
Histology			
Mucinous cancer	14 (33.3)	179 (13.8)	0.000[2]
Signet ring cell cancer	3 (7.1)	22 (1.7)	0.010[3]
Papillary adenocarcinoma	5 (11.9)	237 (18.3)	
Tubular adenocarcinoma	17 (40.5)	732 (56.6)	
Undifferentiated adenocarcinoma	1 (2.4)	6 (0.5)	
Adenosquamous cancer	0 (0)	2 (0.2)	
Adenocarcinoma (unclassified)	2 (4.8)	115 (8.9)	
Differentiation			0.000
Well	2 (9.5)	276 (21.3)	
Moderate	14 (33.3)	630 (48.7)	
Poor	25 (28.6)	203 (15.7)	
Undifferentiated	1 (2.4)	184 (14.2)	
Stage T			0.264
Tis	0 (0)	17 (1.3)	
T1	1 (2.4)	41 (3.2)	
T2	4 (9.5)	232 (17.9)	
T3	16 (38.1)	526 (40.7)	
T4	21 (50.0)	477 (36.9)	
Number of metastatic lymph nodes			0.001
0	11 (26.2)	669 (51.7)	
1-3	10 (23.8)	337 (26.1)	
> 4	15 (35.7)	184 (14.2)	
Nx	6 (14.3)	103 (8.0)	
Distant metastasis			0.021
M0	30 (71.4)	1094 (84.6)	
M1	12 (28.6)	99 (15.4)	
AJCC stage			0.001
0	0 (0)	17 (1.3)	
I	1 (2.4)	221 (17.1)	0.008[4]
II	9 (21.4)	427 (33.0)	
III	19 (45.2)	428 (33.1)	
IV	13 (31.0)	200 (15.5)	0.007[5]
Approach of surgery			0.028
Radical surgery	28 (66.7)	1084 (83.8)	
Palliate surgery	10 (23.8)	151 (11.7)	
Unresectable	4 (9.5)	58 (4.5)	

[1]Rectum *vs* other sites of tumor; [2]Mucinous cancer *vs* other histological types; [3]Signet ring cell cancer *vs* other histological types; [4]Stage Ⅰ *vs* other stages; [5]Stage Ⅳ *vs* other stages. Tis: Tumor *in situ*; NS: Non-significance.

DISCUSSION

A total of 1335 patients with colorectal cancer were ana-

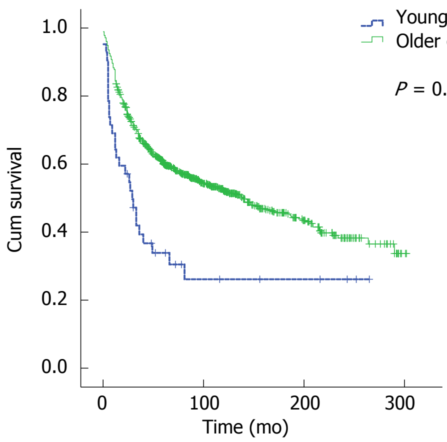

Figure 1 Overall survival of patients in the younger group (≤ 30 years) and older group (> 30 years). The younger group had worse prognosis than the older group (P = 0.000).

Table 2 Multivariate analysis (Cox proportional hazard model) of prognostic factors for 1335 patients with colorectal cancer

Variable	OR	95%CI	*P* value
Stage (Ⅲ + Ⅳ/ Ⅰ + Ⅱ)	2.196	1.827-2.639	0.000
Approach of surgery (non-radical/radical)	4.496	3.718-5.437	0.000
Age (> 30 yr/≤ 30 yr)	0.866	0.592-1.269	0.461
Gender (male/female)	0.997	0.852-1.167	0.970
Tumor location (rectum/colon)	1.270	1.084-1.488	0.003
Differentiation (moderate + well/low)	0.802	0.650-0.990	0.041
Histology (others/mucinous)	0.791	0.632-0.990	0.041

lyzed retrospectively in this study, including 42 (3.1%) patients in the younger group (aged ≤ 30 years). In other studies, the incidence rate was less than 1% and 3% if young patients with colorectal cancer were defined as those aged ≤ 30 years[17-19] and ≤ 40 years[4,15], respectively. The incidence rate in this study was higher than in other regions, suggesting an obvious regional difference. In this study, Eastern China refers to Yangtze River delta region where people enjoy a similar lifestyle and economic status. Consequently, the epidemiological characteristics of colorectal cancer in this region are similar. Therefore, data from our center could represent the features of this tumor in Eastern China. There might be statistical biases about the incidence rate of colorectal cancer in young patients because the data were collected retrospectively by a single medical center.

Gender

There was no significant difference in gender ratio between the two groups. The percentage of female patients is becoming higher with the trend of younger age in gastric cancer. This phenomenon was not seen in colorectal cancer. Estrogen was considered to be related with gastric cancer in younger patients[20]. It is not clear whether estrogen was related to the occurrence of colorectal cancer in young people[21,22]. On the other hand, this study indicated that female patients with colorectal cancer had better outcome than male patients, but with no significant difference (OR = 0.969, P = 0.708) in survival as shown by the multivariate analysis.

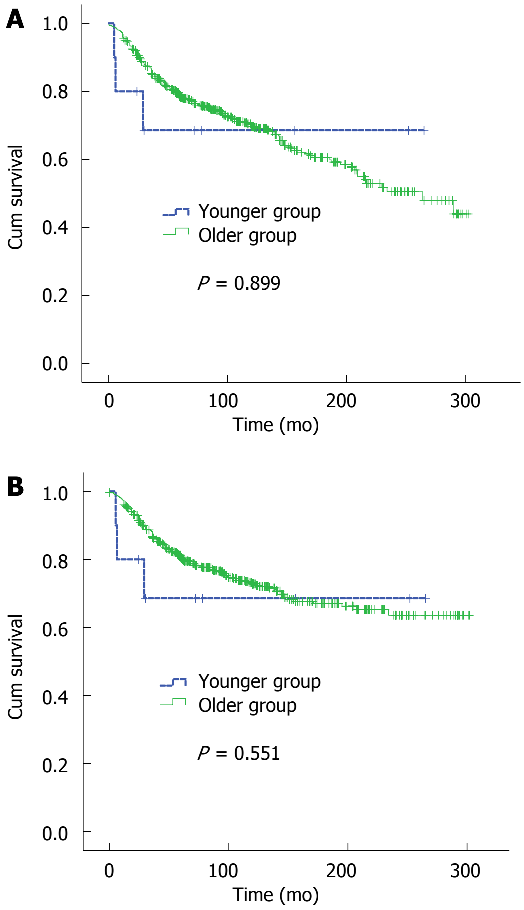

Figure 2 Overall survival of younger patients (≤ 30 years) and older patients (> 30 years) in stage Ⅰ and Ⅱ tumor subgroup. A: Overall survival was totally similar between the two groups (P = 0.899); B: Cancer-related survival was similar between the two groups (P = 0.551).

Tumor location

In this study, the rectum and sigmoid were common sites of the tumor in both groups. The proportion of rectal cancer was higher in the younger group (59.5%) than in older group (49.3%), but without significant difference (P = 0.191). Some reports indicated that the rate of rectal cancer in younger population was higher than in older one[2]. That might be related to the epidemics of colorectal cancer that rectal cancer is more common than colonic cancer in China. Eating habit and lifestyle might contribute more to the occurrence of colorectal cancer than age.

Pathological characteristics

In this study, mucinous tumor and signet ring cell cancer were more common in the younger group than in the older group. A majority of patients in the younger group had poor histologic grade compared with the older group. Studies on gastric cancer also indicated that there were more poorly differentiated cancers in younger

Table 3 Survival of subgroup patients by stratified analysis with stage and approach of surgery

	Age (yr)	*n*	5-yr OS	10-yr OS	Median survival time (mo, 95%CI)
Total[1]	≤ 30	42	33.90%	26.10%	29.0 (18.0-40.0)
	> 30	1293	60.10%	52.20%	140.0 (111.6-168.4)
Stage Ⅰ and Ⅱ	≤ 30	10	68.60%	/[4]	/[4]
	> 30	665	78.60%	69.80%	264.0 (203.5-324.5)
Stage Ⅲ and Ⅳ[2]	≤ 30	32	24.60%	14.80%	22 (2.6-41.4)
	< 30	628	40.40%	33.30%	35 (27.9-42.1)
Radical surgery[3]	≤ 30	28	44.30%	34.20%	40.0 (10.1-69.9)
	> 30	1082	69.60%	60.50%	/[5]
Non-radical surgery	≤ 30	14	14.30%	0%	6 (2.3-9.7)
	> 30	211	11.80%	0%	11 (9.2-12.8)

[1]χ^2 = 14.146, *P* = 0.000; [2]χ^2 = 5.101, *P* = 0.024; [3]χ^2 = 7.830, *P* = 0.005; [4]The sample was too small to analyze; [5]The median survival time was not reached.

population than in older population, especially signet ring cell cancer[23]. It was not clear about the age impact on the occurrence of gastrointestinal cancer.

Stage

Compared with the older population, the percentage of patients with stage Ⅳ disease increased and that of patients with stage Ⅰ disease decreased in the younger group. As for the infiltration of tumor and nodal metastasis, the patients in the younger group presented with more aggressive findings. Some studies found that 66.0% of younger patients with colorectal cancer were diagnosed with stage Ⅲ or Ⅳ disease, which was obviously lower (32.0%) in the older patients[24]. This may result from the poor differentiation and high aggressiveness of tumors which were often diagnosed in younger patients with colorectal cancer. Besides, younger patients with colorectal cancer often had delayed diagnosis, but the older ones would be diagnosed earlier through screening program.

Overall survival

Univariate analysis revealed that the patients in the younger group had poorer survival than those in the older group. The impact of young age on the prognosis of colorectal cancer is not confirmed. Some studies showed that young patients with colorectal cancer had more mucinous cancer and signet ring cancer, poorer histologic grade, later stage and worse prognosis[2,7-9]. But results were contradictory from other studies which indicated that young age had no impact on the prognosis[10-15]. In our study, young colorectal cancer patients had worse prognosis, while multivariate analysis indicated that age was not an independent factor for prognosis. Furthermore, multivariate analysis also showed that disease stage and approach of surgery were strongly related to the prognosis. Worse prognosis might result from stage Ⅲ and Ⅳ disease and non-radical surgery. Therefore, stratified analyses with these two factors were carried out.

The result of stratified analysis with stage indicated that younger patients had poor prognosis, and univariate analysis showed that younger patients presented with mainly stage Ⅲ and Ⅳ disease. The reasons might be that young patients had more poorly differentiated tumor, and mucinous carcinoma and signet ring cell cancer, which were more aggressive in the same stage. As for patients with stage Ⅰ and Ⅱ disease, age exerted no effect on the survival. In this study, there were more patients in the older group who did not die of colorectal caner. In order to exclude the influence of the non-cancer special death, the cancer-related survival in patients with stage Ⅰ and Ⅱ disease was analyzed. The result showed no significant difference in cancer-related survival in stage Ⅰ and Ⅱ tumor between the two groups. The study of Quah *et al*[25] considered that patients with an earlier stage disease had better survival in younger group than older group; young patients were more tolerable to surgery and aggressive adjuvant chemotherapy and radiotherapy[26]. And the study of McMillan *et al*[27] indicated that in the older group, non-special cancer factors were major causes of death.

The stratified analysis with approach of surgery revealed that patients had poorer prognosis in the younger group than in the older group with radical surgery, but there was no significant difference between the two groups without radical surgery. In stratified analysis with stage, patients with stage Ⅰ and Ⅱ disease had similar prognosis between the two groups. Stage Ⅰ and Ⅱ tumors were often considered to be resectable. For patients with resectable stage Ⅲ and stage Ⅳ tumors, younger age strongly contributed to poor survival. For patients who received operation without adjuvant chemotherapy in the 1980s and 1990s, the value of postoperative adjuvant therapy should be highlighted for patients with resectable stage Ⅲ or more advanced colorectal cancer[28].

The current study had some limitations. The clinical data did not include the signs and symptoms of colorectal cancer patients. It was impossible to identify the alarming symptoms for younger patients. Family histories were not described, which were routinely detected in young population as the other studies[29]. The percentage of lost patients was 20%, which might influence the result of survival. In China, there are several medical centers owning elaborate clinical data, but few centers carried out the systemic follow-up. The data of 10-year follow-up are rare.

In summary, compared with older patients, the younger ones have specific clinicopathologic characteristics that

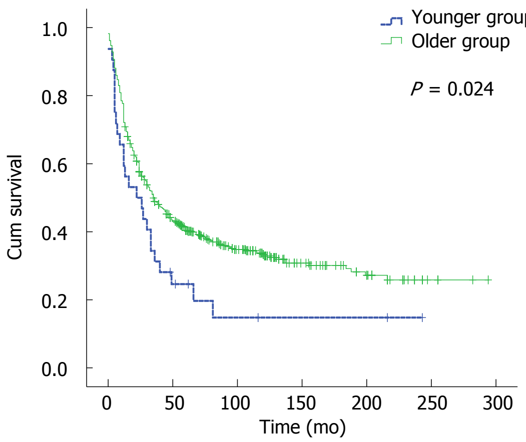

Figure 3 Overall survival of younger patients (≤ 30 years) and older patients (> 30 years) in stage Ⅲ and Ⅳ tumor subgroup. The younger group had worse prognosis than the older group (P = 0.024).

are worthy to be explored and managed differentially. Younger patients with colorectal cancer tend to be diagnosed at later stage. For younger patients who have poor survival, especially those with stage Ⅲ and Ⅳ disease and treated by radical surgery, more aggressive adjuvant therapies are recommended.

COMMENTS

Background

The incidence rate of colorectal cancer has been increasing in recent years. The onset age of colorectal cancer is getting younger. Should the young colorectal cancer patients be treated as a heterogeneous group? It is important to explore the phenotype of young patients with colorectal cancer.

Research frontiers

Age is an independent prognostic factor for many cancers such as breast cancer, thyroid cancer and gastric cancer. Young patients have more triple negative breast cancers and worse prognosis. Lymph node-positive thyroid cancers are commonly diagnosed in adolescent patients, who have satisfactory prognoses. Young patients with gastric cancer in early stage have better prognosis than old ones, while their prognoses are worse in advanced gastric cancer. It is unknown about the age impact on the prognosis of colorectal cancer. Some studies showed that young patients with colorectal cancer had more mucinous cancer and signet ring cancer, poorer histologic grade, later stage and worse prognosis. But results were contradictory in other studies which indicated that the young age had no impact on prognosis.

Innovations and breakthroughs

The authors described systematically for the first time the clinical characteristics and prognosis of young colorectal cancer patients in Eastern China. The incidence rate of young colorectal cancer was higher than in other reports. Colorectal cancer in younger patients was characterized by poorer differentiation and advanced stage. Young colorectal cancer patients had worse prognosis, especially those with stage Ⅲ and Ⅳ disease, rather than stage Ⅰ and Ⅱ disease.

Applications

Relapse risk of postoperative stage Ⅱ colonic cancer is a crucial factor for decision-making in postoperative treatment. This study showed that age was not an independent risk factor for stage Ⅱ colorectal cancer. On the other hand, young colorectal cancer patients with stage Ⅲ and Ⅳ disease had worse prognosis, and more aggressive adjuvant therapy is recommended for these patients.

Terminology

Young patients with colorectal cancer: Onset age of colorectal cancer was less than or equal to 30 years. Eastern China refers to Yangtze River delta region where people have similar lifestyle and economic conditions. Epidemiological characteristics of colorectal cancer in this region are also similar.

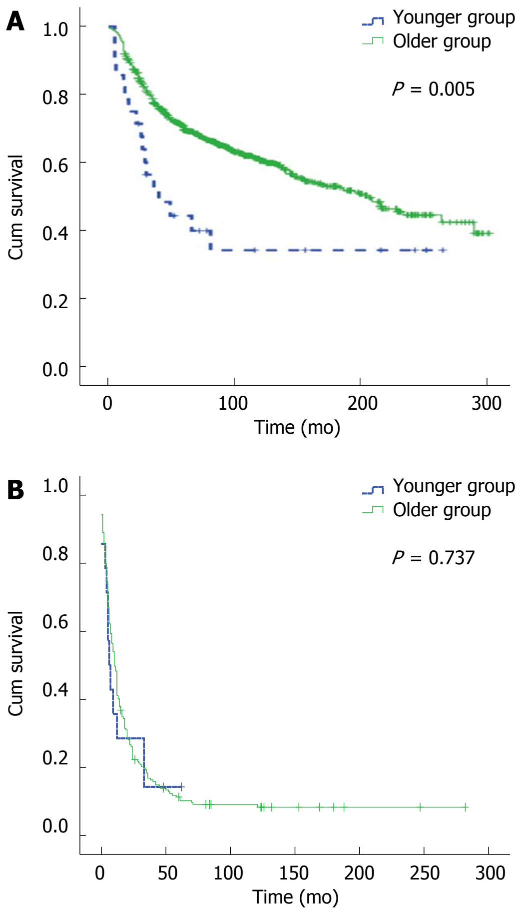

Figure 4 Overall survival of younger patients (≤ 30 years) and older patients (> 30 years) based on approach of surgery. A: The younger group had worse prognosis than the older group (P = 0.005) undergoing radical surgery; B: There was no difference between the two groups (P = 0.737) treated by non-radical surgery.

Peer review

The study described the detailed clinicopathologic characteristics of 1335 cases of colorectal cancer in Eastern China and analyzed the significance of prognosis by many statistical methods. The major goal of authors was to analyze the difference between younger patients (≤ 30 year-old) and older patients (> 30 year-old). The information enclosed in this manuscript is very plentiful and clear, and the authors applied many different statistical methods to perform the analysis. Although the results are not novel and methodology was orthodox, it is worth reporting the present results.

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BRIEF ARTICLE

Clinical effects and complications of TIPS for portal hypertension due to cirrhosis: A single center

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Abstract

AIM: To determine the clinical effects and complications of transjugular intrahepatic portosystemic shunt (TIPS) for portal hypertension due to cirrhosis.

METHODS: Two hundred and eighty patients with portal hypertension due to cirrhosis who underwent TIPS were retrospectively evaluated. Portal trunk pressure was measured before and after surgery. The changes in hemodynamics and the condition of the stent were assessed by ultrasound and the esophageal and fundic veins observed endoscopically.

RESULTS: The success rate of TIPS was 99.3%. The portal trunk pressure was 26.8 ± 3.6 cmH_2O after surgery and 46.5 ± 3.4 cmH_2O before surgery (P < 0.01). The velocity of blood flow in the portal vein increased. The internal diameters of the portal and splenic veins were reduced. The short-term hemostasis rate was 100%. Esophageal varices disappeared completely in 68% of patients and were obviously reduced in 32%. Varices of the stomach fundus disappeared completely in 80% and were obviously reduced in 20% of patients. Ascites disappeared in 62%, were markedly reduced in 24%, but were still apparent in 14% of patients. The total effective rate of ascites reduction was 86%. Hydrothorax completely disappeared in 100% of patients. The incidence of post-operative stent stenosis was 24% at 12 mo and 34% at 24 mo. The incidence of post-operative hepatic encephalopathy was 12% at 3 mo, 17% at 6 mo and 19% at 12 mo. The incidence of post-operative recurrent hemorrhage was 9% at 12 mo, 19% at 24 mo and 35% at 36 mo. The cumulative survival rate was 86% at 12 mo, 81% at 24 mo, 75% at 36 mo, 57% at 48 mo and 45% at 60 mo.

CONCLUSION: TIPS can effectively lower portal hypertension due to cirrhosis. It is significantly effective for hemorrhage of the digestive tract due to rupture of esophageal and fundic veins and for ascites and hydrothorax caused by portal hypertension.

Key words: Transjugular intrahepatic portosystemic shunt; Cirrhosis; Portal hypertension; Therapeutic effect; Complication

Core tip: This study identified the clinical effects and complications of transjugular intrahepatic portosystemic shunt (TIPS) for portal hypertension due to cirrhosis in 280 patients who underwent this procedure at our centre between January 2005 and December 2009. TIPS can effectively lower portal hypertension due to cirrhosis. It is significantly effective for hemorrhage of the

digestive tract due to rupture of esophageal and fundic veins and for ascites and hydrothorax caused by portal hypertension.

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is an effective procedure for portal hypertension due to cirrhosis and related complications. At the end of the 1980s, Rösch *et al*[1] and Rössle *et al*[2] first reported the use of Palmaz, a self-expanding stent. Since then Palmaz had been gradually applied and disseminated in clinical practice. In our centre, TIPS was used, in the initial stage, mainly for the treatment of patients intolerant of surgery, patients with recurrent hemorrhage despite medication and in patients with refractory ascites. As this procedure was developed and improved, it was also used in the treatment of recurrent hemorrhage of the digestive tract due to cirrhosis, hemorrhage after endoscopic ligation and sclerosing therapy, hemorrhage after surgery, portal thrombosis, ascites and hydrothorax due to portal hypertension, hepatorenal syndrome, and emergency hemorrhea. In this study, the significant clinical effects and complications of TIPS are discussed in 280 patients who underwent this procedure at our centre between January 2005 and December 2009.

MATERIALS AND METHODS

Patients

The clinical data on the outcome of TIPS in 280 patients between January 2005 and December 2009 were retrospectively analyzed. These 280 patients with portal hypertension due to cirrhosis met the criteria of the American Hepatological Association[3,4] for the clinical application of TIPS.

TIPS procedure

Patients with cirrhotic portal hypertension underwent routine abdominal enhanced computed tomography (CT) scanning and hepatic portal vein CT three-dimensional reconstruction prior to TIPS. During TIPS, after paracentesis from the right hepatic vein or hepatic segment of the inferior vena cava to the branch of the portal vein, direct portography was carried out, then balloon dilatation, followed by stent placement. Portal venous pressure was measured before and after stent placement. Spring wire loops, a gelatine sponge and sclerosing agent were used for blockage of the collateral circulation of esophageal and fundic varices. The stents used were Zilver stents. Specifications of the stents: ZIV 6-80-8 or 10-8.0 (Cook Corporation, Bloomington, IN). Specifications of the balloon: ATB 5-35-8-6.0 or 4.0 (Cook Corporation). Specifications of the spring wire loop: MWCE-35-3-3, 4, 5, 8, 10 (Cook Corporation). Generally, the puncture path was dilated with a balloon of 8 mm inside diameter, and a stent of 8 or 10 mm inside diameter was then positioned.

Postoperative management

Anticoagulant therapy was administered in addition to routine expectant treatment. Heparin sodium 12500 IU was administered by in intravenous drip 24-h for 7 d 24 h after surgery, and then oral sodium warfarin tablets for 1 year. Prothrombin time (PT) was maintained for 17-20 s.

Follow-up

All patients were followed up 1 wk and 1 mo after surgery, followed by every 3 mo for 12 mo and then every 6 mo after 12 mo. Each follow-up visit included ultrasonography, liver and renal function tests, blood ammonia, routine blood examination and blood coagulation tests, and symptoms and signs of portal hypertension. Gastroscopy was performed in each patient from the month 1 to the month 3 after surgery and direct portography from the month 9 to the month 12.

Statistical analysis

All measurement data are presented as mean ± SD. The data before and after surgery were analyzed using the t test. P values < 0.05 were considered statistically significant. Stent stenosis, hepatic encephalopathy, recurrent hemorrhage and survival were analyzed by the Kaplan-Meier method.

RESULTS

Clinical data

All 280 patients had portal hypertension. Of these patients, 220 had severe esophageal varices, 60 had severe esophageal and moderate-severe fundic varices, 42 had a large amount of ascites, 31 had a moderate amount of ascites and 40 had intractable ascites which was complicated by a large right hydrothorax in 4. Table 1 shows the patients'sex, causes of portal hypertension and Child-Pugh grading. Differences between the patients' sex, age, causes and Child-Pugh grading and their survival rate, incidence of rebleeding, incidence of hepatic encephalopathy and incidence of stent stenosis were not statistically significant ($P > 0.05$).

TIPS procedure

All 280 patients underwent puncture of the right internal jugular vein. Of these patients, 200 underwent puncture of the right hepatic vein, 80 puncture of the inferior vena cava near the liver, 198 underwent puncture of the right branch of the portal vein and 80 puncture of the left branch and 2 had severe hemorrhage in the abdominal cavity during this procedure (1 died, and the other

Table 1 Clinical data on transjugular intrahepatic portosystemic shunt in 280 patients

Clinical factor	No. of patients
Sex	
Male	223
Female	57
Age (yr, mean ± SD)	48.2 ± 13.7
Procedure	
Elective	260
Emergency	20
Indication for TIPS	
Hemorrhage of upper digestive tract	265
Hepatorenal syndrome	15
Cause	
Cirrhosis after hepatitis B virus infection	168
Cirrhosis after hepatitis C virus infection	10
Hepatatis B virus infection complicated by schistosomial cirrhosis	16
Hepatitis B virus infection complicated by alcoholic cirrhosis	54
Alcoholic cirrhosis	24
Unexplained cirrhosis	8
Child-Pugh grading	
A	60
B	184
C	36

TIPS: Transjugular intrahepatic portosystemic shunt.

Table 2 Changes in dynamics and diameters of blood vessels before and after transjugular intrahepatic portosystemic shunt (n = 278, mean ± SD)

	Preoperative	Postoperative	P value
Portal venous pressure (cmH_2O)	46.5 ± 3.4	26.8 ± 3.6	< 0.001
Portal venous internal diameter (cm)	1.68 ± 0.15	1.32 ± 0.11	0.007
Splenic venous internal diameter (cm)	1.31 ± 0.05	1.12 ± 0.03	0.009
Blood velocity in the portal vein (cm/s)	15.2 ± 4.7	49.3 ± 18.5	< 0.001
Blood velocity in the shunt pathway (cm/s)		154.0 ± 32.6	

All parameters shown in Table 1 were significantly different before and after surgery (P < 0.01).

survived after emergency treatment). The success rate of surgery was 99.3 % and the incidence of lethal complications was 0.7%. Embolism caused a collateral circulation in esophageal and fundal varices.

Influence of TIPS on liver hemodynamics

Following the establishment of a portosystemic shunt pathway, liver hemodynamics changed. Portal pressure decreased after surgery, the internal diameters of the portal and splenic veins decreased and the blood velocity in the trunk of the portal vein increased (Table 2).

Liver function before and after TIPS

Liver function was slightly altered after TIPS. No marked changes in alanine aminotransferase (ALT), total bilirubin,

Table 3 Changes in liver function and prothrombin time before and after transjugular intrahepatic portosystemic shunt (n = 278, mean ± SD)

TIPS	ALT (IU/L)	TBIL (μmol/L)	Alb (g/L)	PT (s)
1 wk before TIPS	40.78 ± 5.41	29.33 ± 5.97	32.49 ± 5.14	13.43 ± 1.44
1 mo after TIPS	42.26 ± 2.32	28.45 ± 8.71	33.25 ± 4.18	17.73 ± 1.83[a]
P value	0.679	0.813	0.716	0.036

[a]P < 0.05 *vs* the preoperative data. ALT: Alanine aminotransferase; TBIL: Total bilirubin; Alb: Albumin; PT: Prothrombin time; TIPS: Transjugular intrahepatic portosystemic shunt.

and albumin (Alb) before and after surgery were observed. Routine anticoagulant therapy was given postoperatively. PT increased significantly after surgery (Table 3).

Clinical effects of TIPS

The short-term hemostasis rate was 100% when TIPS was used in the treatment of emergency hemorrhage and recurrent hemorrhage unresponsive to medication, endoscopy or surgery. Ascites disappeared completely in 62% of patients, decreased obviously in 24% and remained in 14%. The total effective rate was 86%. Hydrothorax completely disappeared in 100% of patients. Fifteen patients who had hepatorenal syndrome became responsive to diuretic therapy. Ascites completely disappeared in 7 patients and was obviously reduced in 8 after 7-14 d of observation.

Complications of TIPS

Complications occurred during surgery and both short- and long-term postoperative complications were observed (Table 4). The most serious complication was abdominal cavity hemorrhage, which frequently endangered the patient's life. Short-term severe complications after surgery were hepatic failure, septicemia and abdominal cavity hemorrhage. Intermediate and long-term complications were stent stenosis and hepatic encephalopathy.

Follow-up

All the 278 patients who underwent TIPS were followed up. Hemorrhage, stent function, hepatic encephalopathy and survival were observed during the follow-up. The incidence of recurring hemorrhage was 9% in 12 mo, 19% in 24 mo and 35% in 36 mo (Figure 1A). The incidence of stent stenosis was 24% in 12 mo and 34 % in 24 mo postoperatively (Figure 1B). The incidence of hepatic encephalopathy was 14% in 3 mo, 17% in 6 mo and 19% in 12 mo (Figure 1C). The cumulative survival rate was 86% in 12 mo, 81% in 24 mo, 75% in 36 mo, 57% in 48 mo and 45% in 60 mo (Figure 2). In our center, 3 patients died 1 mo after TIPS, of whom 2 died of hepatic failure and 1 of septicemia.

DISCUSSION

TIPS is an effective method of treating portal hyperten-

Table 4 Complications of transjugular intrahepatic portosystemic shunt (n = 280) n (%)

Complication	
Intraoperative	
Abdominal cavity hemorrhage	2 (0.7)
Puncture of biliary tract	10 (3.6)
Puncture of gallbladder	5 (1.8)
Puncture of hepatic artery	8 (2.9)
Puncture of hepatic capsule	18 (6.4)
Heterotopic embolism	4 (1.4)
Displacement of stent	6 (2.1)
Short-term after TIPS (1 mo)	
Abdominal cavity hemorrhage	2 (0.7)
Hepatic failure	20 (7.2)
Hemorrhagic ascites	7 (2.5)
Hemorrhage of digestive tract	4 (1.4)
Septicemia	3 (1.1)
Hemolysis	8 (2.9)
Hyperglycemia	4 (1.4)
Hemobilia	2 (0.7)
Subcapsular hematoma of liver	3 (1.1)
Puffiness of face	2 (0.7)
Long-term after TIPS (> 1 mo) cumulative incidence	
Stent abnormality	
12 mo	24%
24 mo	34%
Hepatic encephalopathy	
3 mo	14%
6 mo	18%
12 mo	19%

TIPS: Transjugular intrahepatic portosystemic shunt.

sion due to cirrhosis and its complications. Because it is characterized as safe, micro-traumatic, effective and easily repetitive, it has been used more and more widely in clinical practice. Hepatic transplantation has not yet been popularized in China, therefore TIPS is effective for treating portal hypertension due to cirrhosis and its complications, particularly hemorrhage of the digestive tract, and is effective for treating refractory ascites and hydrothorax caused by portal hypertension. It is used mainly in the treatment of approximately 15%-20% of patients with refractory ascites and hemorrhage due to varices that are not responsive to medication or endoscopy. TIPS is used for 99% of cases with these two conditions[5,6]. In addition, TIPS is used for the treatment of hepatic hydrothorax, hepatorenal syndrome, hepatopulmonary syndrome and Budd-Chiari syndrome[7]. In our study, TIPS was also successfully adopted in emergency and portal thrombosis.

In our study, the success rate of TIPS was 99.3%. Once the shunt pathway was established, the portal vein pressure fell from 46.5 ± 3.4 cmH_2O before surgery (P < 0.01) to 26.8 ± 3.6 cmH_2O after surgery. The instant rate of hemostasis was 100%. These results are consistent with literature reports[8-10] which show that the short-term effective rate of TIPS is 90%-97.4% and the rate of emergency hemorrhage control is 90%-100%. In the present study, the total effective rate of TIPS for ascites was 86% and the rate of elimination of hydrothorax was

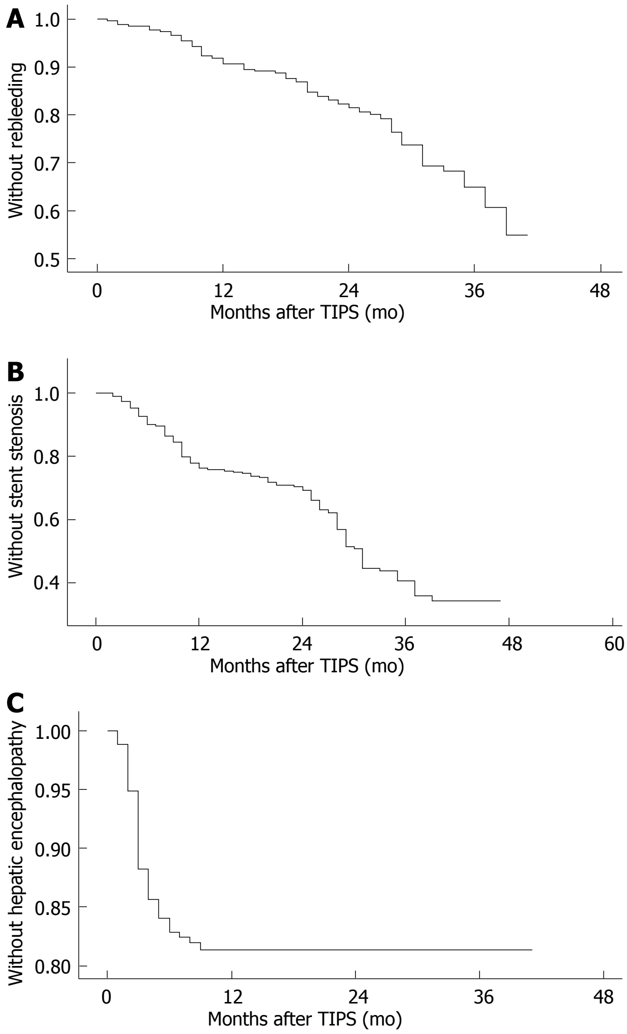

Figure 1 Incidence of recurring hemorrhage (A), stent stenosis (B) and hepatic encephalopathy (C) after transjugular intrahepatic portosystemic shunt.

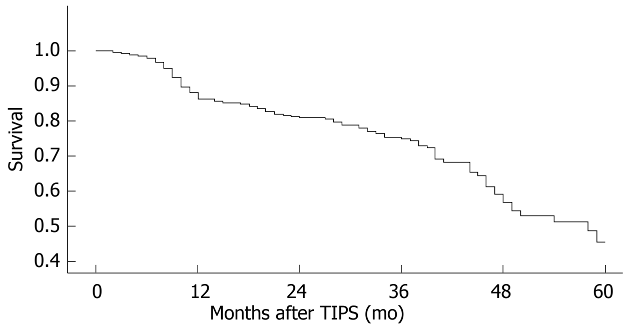

Figure 2 Cumulative survival rate after transjugular intrahepatic portosystemic shunt.

100%. These findings are similar to the reported[9] effective rate of 50%-92% for refractory ascites and elimination of ascites in 70%-75% of patients. According to the literature reports[11]: 82% of patients who underwent TIPS had significantly reduced hydrothorax and in 71% hydrothorax was eliminated, however, patients over 60 did not respond well to TIPS. In our study, hydrotho-

rax was eliminated in 4 cases. However, the number of cases was small and the therapeutic effects remain to be determined. Fifteen patients with hepatorenal syndrome became responsive to diuretic therapy after TIPS and their renal functions were obviously improved. Ascites was eliminated in 7 of these patients and was improved in 8. Eight patients were alive after a one-year of follow-up (53%). According to the literature reports[12], renal function was remarkably improved by TIPS in patients with hepatorenal syndrome and the survival rate was 48% after a one-year follow-up, however, only 10% of the patients who did not undergo TIPS lived for three months. These findings suggest that TIPS is an effective method of treating hepatorenal syndrome.

Of the 280 patients in the present study, 2 had abdominal cavity hemorrhage after TIPS. One patient died and the other survived after portal vein repair. Hemorrhage was due to dilation of the sacculus near the bifurcation of the portal vein. The incidence of this severe complication was 0.7%. It is the most severe complication of TIPS in that a patient immediately suffers from hemorrhagic shock and dies. Therefore, the operator should pay close attention to this complication. As reported[13], the incidence of lethal complications related to the procedure was 0.6%-4.2%. The most critical complications after TIPS were worsening of liver function and hepatic encephalopathy. Both were related to a decrease in blood perfusion in the liver due to the establishment of the shunt pathway[14]. In our study, various degrees of hepatic injury occurred in all 280 patients after TIPS, however liver function was gradually restored after approximately 1 mo in most patients. The changes in bilirubin, ALT and Alb were not significantly different. This may be related to our patients having mainly Child-Pugh B and A liver function, few patients with Child-Pugh C liver function (36 patients) and dilation of the path using a balloon of 8 mm inside diameter. The incidence of hepatic failure 1 mo postoperatively was 7.2% (20/278) in our patients, which occurred mainly in emergency and Child-Pugh C TIPS patients. This may be related to poor liver reserve function in some patients and hypoperfusion of the liver due to the artificial shunt and the short supply of hepatic nutrients.

In order to reduce and avoid severe complications of TIPS, the operator is required to be familiar with the anatomy of the portal system. As reported[15,16], the bifurcation of the portal vein is in the liver in about 25.8% of patients, outside the liver in about 48.4% and in the hepatic capsule in about 25.8%. In patients with cirrhosis, the cleavage of the liver is widened and the right trunk and the left horizontal trunk are outside the parenchyma of the liver with bare inferior walls, suggesting that puncture of the bifurcation and peri-bifurcation region is very dangerous. Therefore, the puncture point should be located 2 cm above the bifurcation of the portal vein to reduce or avoid the risk of hemorrhage due to portal vein rupture. In addition, the blood coagulation mechanism is poor in some patients, especially if they have ascites. Hemorrhage will occur if the hepatic capsule is ruptured, and is not easy to stop. Of our patients, 2 (0.7%) had postoperative abdominal cavity hemorrhage and 7 (2.5%) bloody ascites. The bleeding stopped after management.

The intermediate and long-term complications of TIPS are stent abnormality and hepatic encephalopathy. It is reported[10,17] that the rate of stent abnormality (inclusive of stenosis and obstruction) is 17%-50% 6 mo after TIPS and 23%-87% 12 mo after TIPS. The application of a Viatorr stent has improved the condition.

The current criteria[18-20] for the evaluation of stent abnormalities (mainly stenosis) are: (1) the velocity of blood flow is over 200 cm/s or less than 50 cm/s in the shunt path, or the diameter of the shunt path is less than 50%; (2) the velocity of blood flow is less than 20 cm/s in the portal vein; (3) the portosystemic pressure gradient is more than or equal to 16 cmH_2O; (4) portal hypertension recurs, *i.e.*, esophagofundic hemorrhage due to varicose vein or ascites not responsive to low salt diet therapy and routine diuretic therapy. Once the stent abnormality is detected by ultrasound, direct portography and repair should be carried out.

The cumulative rate of stent stenosis is 24% in 12 mo and 34% in 24 mo. The currently used Viatorr stent-graft was first adopted in Europe at the end of 1999 and granted approval by the FDA in 2004. The technical success rate is 100%. The first and second patency rates in one year were 76%-84% and 98%-100%[21-23], respectively. In our study, the patency rate in one year was 76%, which was similar to the first patency rate in the report. Explanations for this rate are as follows: puncture was through the inferior vena cava near the liver (80 cases), avoiding stenosis induced by puncture of the liver vein; the shunt was straight and short apart from the left portal branch; attention was paid to the appliance of the puncture path and care was taken care to avoid angulating the stent; and anticoagulant therapy was given after the procedure, which lasted 1 year. PT was maintained for 17-20 s. It is now accepted that stent stenosis[24,25] is related to pseudo-endometrial hyperplasia, the mechanism of which is still unclear but leads to active proliferation of myofibroblasts and the accumulation of extracellular matrix containing collagen. The Viatorr stent-graft has not yet been extensively used, therefore, the prevention of stent stenosis is very important. In our centre a pathological study is now being carried out.

Hepatic encephalopathy is another complication of TIPS. In our study, the incidence of hepatic encephalopathy was 14% in 3 mo and 18% in 6 mo after TIPS. According to the literature[8,10,21,23,26], the incidence of hepatic encephalopathy was 33%-55% after TIPS and 13%-26% after therapeutic endoscopy. International reports[27,28] showed that there was no significant difference in the occurrence of hepatic encephalopathy between the bare and Viatorr stents 10 mm in diameter, and the incidence was 20%-30%. The incidence of hepatic encephalopathy was 5%-10% when Viatorr stents of 8 mm in diameter were used, which supported shunting without hepatic

encephalopathy. In our study, the incidence of hepatic encephalopathy was lower than that reported and similar to that of the Viatorr stent and endoscope, which may be related to the selection of patients, puncture paths, stent diameters, etiological treatment and postoperative management. Of the 280 patients, most were graded as Child-Pugh A and B (244/280) with better liver function potential. Cirrhosis was induced mainly by HBV (238/280) and antiviral therapy was given before and after surgery. The patients' general physical condition was improved before surgery as far as possible. A stent with an appropriate diameter was carefully selected to avoid over shunting. Generally, we chose a balloon of 8 mm inside diameter and a stent of 8 mm or 10 mm inside diameter. For all patients, protein intake was limited 1 wk after surgery, bowel movement was regulated and enema with vinegar ordered to prevent intestinal infection. The mechanism of hepatic encephalopathy[29] involves multiple factors, but is mainly related to a decrease in blood flow and enhancement of the biological availability of enteric toxins.

The rate of recurrent hemorrhage was 9% in 1 year and 19% in 2 years after TIPS in our study. From previous reports[10,17,21,30] the rate was 15% in 1 year and 21% in 2 years after TIPS; and was 48% in 1 year and 52% in 2 years after gastroscopic treatment; and was less than 10% with the Viatorr stent. It was believed that the rate of recurrent hemorrhage was higher in the gastroscope group than in the TIPS group; and was lower in the Viatorr stent group than in the bare stent group. In our study, the rate of recurrent hemorrhage was similar to that of the Viatorr stent. Recurrent hemorrhage was related to stent abnormality. Any cause of stent stenosis or obstruction could lead to portal hypertension again, and the obstructed collateral circulation might reopen or a new collateral circulation could appear, resulting in hemorrhage from esophageal or fundic varices. Once the varicose vein ruptures, recurrent hemorrhage occurs. The maintenance of stent function is important in avoiding this situation. In our research, the rate of stent stenosis was low and the rate of recurrent hemorrhage was also low. In addition, the collateral vein with esophageal and fundic varices due to intraoperative embolism could significantly reduce or delay the occurrence of rebleeding.

Gastroscopy was performed in our patients. The results showed that 68% of patients had complete relief of esophageal varices and 32% had obvious relief. Approximately 80% of patients had complete relief of stomach fundic varices and 20% had obvious relief. This confirmed the effectiveness of TIPS and was an important procedure for recurrent hemorrhage. In our patients, hyperglycemia, puffiness of the face and other rare complications occurred in addition to the complications reported. Hyperglycemia may be explained by the metabolic disorder of glucose in the liver and insulin injection is indicated. The cause of puffiness of the face is unclear, but it gradually disappeared following diuretic therapy.

The cumulative survival rate was 86% at 1 year and 81% at 2 years after TIPS in our patients, which was similar to previously published reports[8,9] that is 64%-87% at 1 year and 56%-71% at 2 years after TIPS. Survival is related to liver function reserve. The survival rate was lower in patients graded as Child-Pugh C than in patients graded as A and B. Our patients were mainly Child-Pugh B, and few patients had Child-Pugh A and B. Statistical analysis showed that the survival rate of the patients was not significantly correlated with their Child-Pugh grading of liver function, which requires further study. There were 15 patients with hepatorenal syndrome in our study, with a death rate of 47% 1 year after TIPS. Three patients died 1 mo postoperatively, of whom 2 died of hepatic failure and 1 of hematosepsis. Our patients mainly developed cirrhosis after hepatitis B virus infection. The etiological treatment is critical in that we found that antiviral therapy and moderate shunting prolonged the survival of patients, especially of those graded as Child-Pugh A and B. These findings remain to be confirmed by future multicenter, randomized and controlled trials.

TIPS is characterized by its effectiveness and few complications. However, stent stenosis and hepatic encephalopathy are still leading factors affecting the intermediate and long-term therapeutic effects. Even though these problems can be solved to a considerable degree by the use of the Viatorr stent, this procedure is not popular in China, and the mechanism of stent stenosis remains to be studied further. Therefore the determination of the therapeutic effects and the association of the shunt pathway with encephalopathy requires further research.

COMMENTS

Background

Esophageal and fundic varicose hemorrhage is a critical complication of portal hypertension due to cirrhosis, and often endangers the patient's life. The clinical effects of routine treatment on hepatic thoracicoabdominal ascites and hepatorenal syndrome are not good. Transjugular intrahepatic portosystemic shunt (TIPS) is an ideal method of treating these complications.

Research frontiers

TIPS is one of the most difficult operations in vascular interventional therapy at the present time. A shunt path must be established between the branches of the hepatic veins and portal vein, and at the same time a collateral circulation by embolization in esophageal and fundic varices is necessary to achieve a partial shunt and cutout. TIPS has progressively become the method of choice for treating portal hypertension due to cirrhosis and its complications.

Innovations and breakthroughs

By comparison with the results in the literature, in this study, TIPS improved patient outcome. Portal vein puncture was guided by replacing routine trans-superior mesenteric indirect portal venography with hepatic enhanced computed tomography (CT) scanning and hepatic portal vein CT three-dimensional graphic reconstruction. More cases were treated as the technique was developed. The patients were followed up over a long period, and satisfactory clinical effects were achieved.

Applications

As the authors were unable to carry out hepatic transplantation, the complications of cirrhosis were mainly managed clinically, especially varicose hemorrhage and intractable thoracicoabdominal ascites. The effects of the presently used drugs, endoscopes and surgical management are not ideal, however, treatment by TIPS has achieved satisfactory results. With the constant expansion of indications, TIPS will be used more extensively.

Terminology

TIPS involves the establishment of a shunt path in the liver parenchyma between the two puncture points after paracentesis from the right hepatic vein or

hepatic segment of inferior vena cava to the branch of the portal vein, shunting of the portal venous blood flow and lower portal venous pressure, and at the same time causes a collateral circulation by embolization of esophageal and fundic varices and blockage of hemorrhagic blood vessels.

Peer review

This study comprehensively and systematically evaluated the clinical effects and complications of TIPS for portal hypertension due to cirrhosis, and described how to improve the therapeutic effects of TIPS and the experience in reducing its complications. The improved TIPS is of great clinical significance and favors clinical dissemination.

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BRIEF ARTICLE

"Metroticket" predictor for assessing liver transplantation to treat hepatocellular carcinoma: A single-center analysis in mainland China

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Abstract

AIM: To validate the "Metroticket" predictor using a large cohort of liver transplantation (LT) patients with hepatocellular carcinoma (HCC) in China.

METHODS: In total, 230 cases of LT for HCC treatment at our center, from July 2000 to August 2008, were included in the present study. The predicted 1-, 3- and 5-year post-LT survival rates were calculated using the Metroticket model (http://89.96.76.14/metroticket/calculator/). The predicted and observed long-term survival rates were then compared and analyzed.

RESULTS: The predicted survival rates for all 230 cases, as calculated by the Metroticket model, were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 57.8%, respectively. For the 23 cases with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. For the 42 cases with microvascular invasion but an absence of macrovascular invasion, the predicted 5-year survival rate was 44.9%, and the observed 5-year survival rate was 50%. For the remaining 165 patients without any vascular invasion, the predicted 5-year survival rate was 65.8%, and the observed 5-year survival rate was 66.7%.

CONCLUSION: The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

Key words: Metroticket; Model; Survival; Hepatocellular carcinoma; Liver transplantation

Core tip: The aim of our study was to validate the "Metroticket" predictor using a large cohort of liver transplantation (LT) patients with hepatocellular carcinoma (HCC). The predicted survival rates for all 230 cases, as calculated by the Metroticket model, were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 62.2%, respectively. For the 23 cases with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally[1], and this burden is heavier in China, which accounts for nearly 55% of all cases worldwide[2]. Despite the prevalence of using the hepatitis B vaccine in recent years, HCC is also the fifth most common malignancy in males and the sixth most common in females in China[3]. Liver transplantation (LT), resection and radiofrequency ablation (RFA) were once the only three potential curative treatments for early HCC[4]. LT was theoretically the best therapeutic option for HCC patients due to the procedure's overall eradication of the remnant liver with cirrhosis compared with resection and RFA[5,6]. Despite its thoroughness, LT was not suitable for all HCC cases: in that time, the very low survival rate after LT in HCC patients was mainly due to advanced HCC[7]. The Milan criteria, which were proposed in 1996 by Mazzaferro *et al*[8], resulted in excellent survival, with a 5-year survival rate of 61.1% compared with the previously observed 5-year survival rate of 25.3% in 1987. Thereafter, dozens of inclusion criteria were introduced for HCC-related LT[9-13]. However, these criteria were only inclusion criteria and could not be used to predict the results of LT, and especially the survival and recurrence rates.

In recent years, many groups have found certain risk factors that predict survival and recurrence after LT in HCC patients[14-18]. However, only few researchers have found risk factors for HCC recurrence after LT and built predictive models, such as the Metroticket[19], Alpha-fetoprotei (AFP)[20] and Markov[21] models. Derived from the largest collection of pathological data from patients with HCC (1556 overall and 1112 exceeding the Milan criteria), the Metroticket model offers individualized survival predictions based on a continuum of tumor size and number, whereby each patient is assigned an individual prognosis for 3- and 5-year survival[22]. The Metroticket model has been validated in several studies[6,21,22]. However, no analysis has been performed on the effectiveness of this predictive model using data from China with a large cohort of HCC cases, where nearly 55% of all cases worldwide[2] occurred and 24801 cases of LT were performed. Thus, in the present study, we aimed to prove the prognostic accuracy of the Metroticket model using single-center data from mainland China.

MATERIALS AND METHODS

Our study used data from a retrospective database on LT in HCC patients that was developed at our center between August 2000 and August 2008 (230 consecutive patients). All of the data from these patients, including baseline demographic data, preoperative laboratory and radiological data, intraoperative data, postoperative recovery data and long-term outcomes, were retrospectively analyzed. All of these data were collected from the China Liver Transplant Registry System. Demographic data included age, gender, height, weight and body mass index (BMI). Preoperative liver function data included underlying liver disease and liver function (Child score and MELD score). Tumor characteristics included the tumor number, diameter and differentiation. Intraoperative data included the graft type (DDLT/LDLT), operative time, blood loss and rate of transfusion. Postoperative data included mortality, complications (classified using the Clavien system), hospital stay days and overall cost. Long-term outcomes were mainly the overall survival rate.

The diagnosis of HCC was confirmed preoperatively in all patients if the patient simultaneously fulfilled the following three criteria: radiological evidence of HCC (helical triple-phase computed tomography or magnetic resonance imaging scans in arterial, portal venous and delayed venous phases; blush with washout; and a pseudocapsule), serology positive for hepatitis B or C and levels of AFP > 400 ng/mL. If the patient lacked one of these features, biopsy (histology or cytology) was performed to prove HCC. For each patient in the present study, a "Metroticket"-predicted survival score was calculated using the online calculator (http://89.96.76.14/metroticket/calculator/). All of the imaging data were based on pre-transplant radiological measurements obtained within 15 d pre-LT. The Metroticket calculator only incorporates tumors greater than 10 mm in diameter and no more than 10 nodules. We also divided all of the patients into subgroups according to the presence of micro- and macrovascular invasion. Thus, the main analysis was a comparison between the Metroticket model-predicted and observed survival rates, and the subgroup analysis also compared the Metroticket model-predicted and observed survival rates in the presence and absence of macrovascular invasion.

All of the deceased donors were brain-dead donors at our hospital, and no prisoners served as donors at our center. All of the liver donations were voluntary and altruistic. Written consent was given by the donors or their families. For all of these procedures, authorization was obtained from the donors' families, the ethics committee and the Red Cross Society of China. The surgical procedure and postoperative antiviral and immunosuppression protocols have been previously reported[23-25].

Descriptive statistics are expressed as proportion for categorical variables, and mean ± SD or median and range were used for continuous variables. The predicted survival rates at 3 and 5 years were calculated using the Metroticket online calculator for each patient, and the mean sum of the individual scores was calculated and compared with our observed survival rates at 3 and 5 years. Overall survival was defined as the time interval between LT and death from any cause. Survival rates were estimated using the Kaplan-Meier method, whereas statistical significance between survival curves was tested by the log-rank test. Statistical tests were considered to be significant when the corresponding P-value was less than 5%. Statistical analyses were performed using the SPSS package (SPSS 17.0, Inc., Chicago, IL).

Table 1 Complications of recipients, as classified by the Clavien system *n* (%)

	LT to treat HCC *n* = 230
Grade Ⅰ: Treated conservatively without any drugs	22 (9.6)
Pleural effusion	8
Wound infection	8
Bile leak	6
Grade Ⅱ: Treated with medication	14 (6.1)
Pneumonia	2
Ascites	2
Bile leak	2
Acute or chronic rejection	6
Hepatic artery thrombosis	2
Grade Ⅲa: Intervention using local anesthesia	25 (10.9)
Hydrothorax	11
Bile leak	6
Ileus	2
Upper gastrointestinal bleeding	3
Intra-abdominal abscess	3
Grade Ⅲb: Intervention using general anesthesia	17 (7.4)
Intra-abdominal Bleeding	6
Biliary obstruction	3
Intra-abdominal abscess	4
Portal venous thrombosis	2
Hepatic artery thrombosis	2
Grade Ⅳa: Single-organ dysfunction	6 (2.6)
Small-for-size syndrome	2
Renal dysfunction	2
Respiratory failure	2
Grade Ⅳb: Multi-organ dysfunction	2 (0.9)
Grade Ⅴ: Death	22 (9.6)
Respiratory failure	3
Graft-*vs*-host disease	1
Cardiopulmonary arrest	2
Liver failure	4
Septic shock	3
Bleeding	3
Rejection	5

LT: Liver transplantation; HCC: Hepatocellular carcinoma.

RESULTS

The baseline demographics of all patients showed that there were many more male patients (210 cases) than female ones (20 cases). The patients' mean age was 46.1 ± 10.3 years, mean height was 165.2 ± 9.1 cm, mean weight was 67.3 ± 8.8 kg and mean BMI was 23.2 ± 2.2 kg/m^2. Underlying liver disease showed that most of these patients (215 cases) were diagnosed with HBV infection. Two patients had HCV, and 13 patients did not have hepatitis B or C. There were 100 patients who were HBV-DNA positive (> 1.00E + 03 copies/mL). The preoperative liver function reflected by the MELD score of these patients was 11.1 ± 5.5 and 129 patients had Child-Pugh A, 66 patients had Child-Pugh B, and 36 patients had Child-Pugh C.

The preoperative imaging scan indicated that the mean diameter of all targets was 8.6 ± 5.0 cm and that the mean target number was 3.1 ± 2.9 for these HCC patients. In total, 26 new tumor targets were found in the explanted liver in 14 patients, and the diameter of these new targets ranged from 0.6 to 2.4 cm. The mean preoperative AFP level was 1838.2 ng/mL: < 400 ng/mL in 97 patients, 400-800 ng/mL in 12 patients, 800-1200 ng/mL in 19 patients, and > 1200 ng/mL in 102 patients. Explanted tumor histopathologic grading indicated 78 patients with good differentiation, 78 patients with moderate differentiation and 74 patients with poor differentiation.

The intraoperative and postoperative data showed that 177 patients had accepted whole-graft LT and that 53 cases had accepted living-donor LT at our center. The mean graft to recipient weight ratio was 0.81 for the 53 DDLT cases. The mean operative time was 7.8 ± 2.1 h, mean blood loss was 874.5 ± 422.5 mL, and mean length of hospital stay was 33.2 ± 12.3 d. Table 1 shows the postoperative complications for all cases. All of these postoperative complications were classified using the Clavien system. The overall complication rate was 47%, the serious (more than grade III) complication rate was 22.5%, and the mortality rate was 9.6% in the hospital.

For all 230 patients, the predicted survival rates calculated by the Metroticket model based on preoperative imaging data were 64.7% and 56.2% at 3 and 5 years, respectively, and the observed survival rates for these patients were 71.3% and 57.8%, respectively. The actuarial 3- and 5-year survival rates were 71.7% (95%CI: 62.3%-77.0%) and 64.8% (53.5%-68.4%), respectively. The Metroticket predictions of the 3- and 5-year survival rates both fell within the 95%CI of the actuarial survival. For the subgroup patients (23 cases) with macrovascular invasion, the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%. For the subgroup patients (42 cases) with microvascular invasion but an absence of macrovascular invasion (as proven by pathological examination), the predicted 5-year survival rate was 44.9%, and the observed 5-year survival rate was 50%. For the patients (165 cases) without macro- or microvascular invasion, the predicted 5-year survival rate was 65.8%, and the observed 5-year survival rate was 66.7%. The most common recurrence site was the liver (78.6%), followed by intra-abdominal metastasis (22.1%), lung metastasis (20.2%), bone metastasis (13.2%) and brain metastasis (4.6%).

DISCUSSION

For HCC patients, LT is one of the most effective treatments. However, there are still continual pressure on limited donor resources, especially in China, and debate about what should be considered as an acceptable minimum survival outcome[22]. Since the first introduction of the Milan criteria for HCC-related LT in 1996, proposed by Mazzaferro *et al*[8], more than one decade of excellent outcomes of LT for HCC treatment was achieved with these restrictive selection criteria. However, many groups worldwide have suggested expanding the Milan criteria due to comparable survival and recurrence outcomes[9-13]. Groups everywhere have suggested adding different types of risk factors for recurrence to the inclusion criteria: for example, Toso *et al*[26] proposed a total volume

of 115 cm[3,11], and Zheng *et al*[27] proposed the AFP level and histological grade. However, most of the criteria only considered the tumor diameter or number alone[10]. The Metroticket calculator was the first to combine the tumor number with the size of the largest nodule and is a model designed to predict 3- and 5-year overall survival after transplantation on the basis of the characteristics of the HCC (the size of the largest nodule, the number of nodules and the presence or absence of vascular invasion) in a given patient. This model changes the paradigm from "one size fits all" to an individual prognosis for each patient[22]. Our key finding is that the Metroticket calculator is an accurate predictor of post-transplant survival for patients with an absence of macrovascular or microvascular invasion, but not for patients with macrovascular invasion.

The Metroticket model was built in 2009 based on data from Europe. These HCC cases were caused by alcoholic or hepatitis C virus-related liver cirrhosis. Raj *et al*[22] tried to evaluate the veracity of this model, but the study cohort was relatively small (82 cases), as mentioned as a weakness in the report, and only 40 cases included HBV. Compared with the small sample size and low rate of HBV cases in Raj's study, our study included 230 cases of HCC-related LT, and nearly all of our cases (93.5%, 215 cases) were HBV cases. Thus, our study may be more reliable and convincing. The Metroticket calculator was derived from explants' pathological data, but many reports[21,22,28] have proven the model's validity based on pre-transplant radiological criteria. Therefore, this model can be applied prospectively to patient selection.

Compared with other inclusion criteria, such as the Milan, Up-to-Seven and UCSF criteria, the Metroticket model provides a continuous range of survival probabilities rather than a dichotomous "in or out" basis for patient selection[22]. The upper limit of the tumor number is 10, and there is no upper limit for tumor diameter; the calculated tumor diameter is the largest one. Most importantly, the model also considers the presence of vascular invasion, which is a very strong risk factor for HCC recurrence after LT. This model considered all of these risk factors when it was built and thus may provide a reliable prediction of outcome for a patient who plans to accept LT for HCC treatment. However, there are certain limitations, as mentioned in Raj's study[22]. The diagnosis of microvascular invasion requires biopsy, with a risk of needle-tract seeding[29] and bleeding and false negatives[30,31]. Several other risk factors are AFP levels[32,33], the neutrophil-to-lymphocyte ratio[15,34] and the serum C-reactive protein[35] and gene[36], and all of these reported risk factors and biomarkers are available before transplantation and can be routinely used to predict recurrence and survival after HCC-related LT.

In the present study, we first examined the effectiveness of the Metroticket model in a subgroup of patients with macrovascular invasion. Our results showed that in subgroup patients with macrovascular invasion, the observed 5-year survival rate was only 8.7%, which was much lower than the predicted 5-year survival rate of 43.5%. It is known that vascular invasion is an independent risk factor for HCC recurrence after LT, especially in the presence of macrovascular invasion[16]. However, there are still certain differences between the effects of macro- and microvascular invasion on HCC recurrence. As mentioned in other studies, macrovascular but not microvascular invasion is a risk factor for HCC recurrence[37,38]. In the present study, we found that the Metroticket model can be used to predict the outcome of microvascular invasion cases but not macrovascular invasion cases. However, the Metroticket calculator website does not make a distinction between micro and macrovascular invasion. Based on our results, we believe that the Metroticket calculator needs revision on the topic of vascular invasion.

Certain potential limitations of this study are related to our single-center data analysis. The need for a 5-year follow-up limited the size of our study, as we could only include patients (230 cases) who received transplants before 2009. The retrospective nature of our study also limited the reliability. In future work, multiple-center, randomized control trials and a larger number of studies may be needed.

In conclusion, with accurately predicted 3- and 5-year survival rates, the Metroticket model should be introduced as a useful tool for selecting HCC patients for LT based on preoperative imaging examinations. However, macrovascular invasion should be considered as a contraindication to use of the Metroticket model.

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COMMENTS

Background

Liver transplantation was theoretically the best therapeutic option for hepatocellular carcinoma (HCC) patients due to the procedure's overall eradication of the remnant liver with cirrhosis compared with resection and radiofrequency ablation. Dozens of inclusion criteria were introduced for HCC-related liver transplantation (LT). However, these criteria were only inclusion criteria and could not be used to predict the results of LT, and especially the survival and recurrence rates. Recent years, many groups have found certain risk factors that predict survival and recurrence after LT in HCC patients. However, only few researchers have found risk factors for HCC recurrence after LT and built predictive models, such as the Metroticket. The Metroticket model offers individualized survival predictions based on a continuum of tumor size and number.

Research frontiers

The Metroticket model has been validated in several studies. However, no analysis has been performed on the effectiveness of this predictive model using data from China. Thus, in the present study, this study aimed to prove the prognostic accuracy of the Metroticket model using single-center data from mainland China.

Innovations and breakthroughs

The Metroticket model was introduced several years ago, but there is still no consensus about its effectiveness. 230 cases of LT for HCC treatment at our center were included in the present study. The predicted 1-, 3- and 5-year post-LT survival rates were calculated using the Metroticket model (http://89.96.76.14/metroticket/calculator/). The predicted and observed long-term survival rates

were then compared and analyzed. Due to the similar predicted and observed long-term survival rates, the Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

Applications

The Metroticket model can be used to accurately predict survival in HCC-related LT cases with an absence of macrovascular invasion.

Terminology

Liver transplantation is a surgical method to cure end-stage liver disease, removing the liver with disease and implanting one or part of new liver from the donor.

Peer review

This is an interesting study to evaluate the effectiveness of the Metroticket model.

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BRIEF ARTICLE

Decreased histone H2B monoubiquitination in malignant gastric carcinoma

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Author contributions: Wang ZJ, Liu C designed the study and wrote the manuscript; Wang ZJ performed the experiments; Yang JL, Wang YP summarized the immunostaining results and analyzed the data; Lou JY, Chen J provided vital reagents and clinical samples; Guo LD wrote the discussion and revised the entire article for intellectual content; all authors have read and approved the final version of the manuscript.

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Abstract

AIM: To investigate H2B monoubiquitination (uH2B) and H3K4 di- and tri-methylation (H3K4-2me, H3K4-3me) levels and their clinical significance in gastric cancer (GC).

METHODS: Immunohistochemistry (IGC) was used to detect the differential levels of uH2B, H3K4-2me and H3K4-3me modifications in GC specimens from chemo/radiotherapy-naïve patients who underwent potentially curative surgical resection (n = 159) and in a random sampling of non-tumor gastric epithelium specimens (normal controls, n = 20). The immunohistochemistry (IHC)-detected modifications were classified as negative, low-level, or high-level using a dual-rated (staining intensity and percentage of positively-stained cells) semi-quantitative method. The relationships between uH2B modification levels and clinicopathological parameters of GC were assessed by a Wilcoxon rank sum test (pairwise comparisons) and the Kruskal-Wallis H test (multiple comparisons). The correlation between uH2B modification and survival was estimated by Kaplan-Meier analysis, and the role of uH2B as an independent prognostic factor for survival was assessed by multivariate Cox regression analysis.

RESULTS: The presence and level of H3K4-2me and H3K4-3me IHC staining was similar between the normal controls and GC specimens. In contrast, the level of uH2B was significantly lower in the malignant gastric tissues (*vs* normal control tissues) and decreased along with increases in dedifferentiation (well differentiated > moderately differentiated > poorly differentiated). The level of uH2B correlated with tumor differentiation (P < 0.001), Lauren's diffuse- and intestinal-type classification (P < 0.001), lymph node metastasis (P = 0.049) and tumor-node-metastasis stage (P = 0.005). Patients with uH2B+ staining had higher 5-year survival rates than patients with uH2B-staining (52.692 ± 2.452 *vs* 23.739 ± 5.207, P < 0.001). The uH2B level was an independent prognostic factor for cancer-specific survival (95%CI: 0.237-0.677, P = 0.001).

CONCLUSION: uH2B displays differential IHC staining patterns corresponding to progressive stages of GC. uH2B may contribute to tumorigenesis and could be a potential therapeutic target.

Key words: Gastric cancer; Epigenetics; Histone modification; H2B monoubiquination; Nuclear immunostaining

Core tip: The abundant H2B monoubiquination (uH2B) modification detected by immunohistochemistry (IHC) in normal human gastric epithelium is decreased in malignant gastric cancer specimens, and the decreasing trend is correlated with decreased tumor differentiation, Lauren's classification intestinal-type, presence of lymph node metastasis, and TNM stage. Positive uH2B staining is associated with higher 5-year survival. Multivariate analysis identified uH2B modification level as an independent prognostic factor for gastric cancer-specific survival. Collectively, these findings indicate the clinical significance of IHC-detected uH2B differential staining patterns as a potential prognostic biomarker in early stage gastric cancer.

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INTRODUCTION

Focused public health efforts to increase awareness of gastric cancer (GC) and implementation of screening programs to detect malignancy in asymptomatic patients have led to a decline in the overall mortality of this disease worldwide. Asian countries continue to report the highest incidence rates of GC and these cases have worse prognosis. The low overall 5-year survival rate of GC cases in China (about 40%)[1,2] highlights the particular burden facing these nations' healthcare systems and the impact on the overall social and economic well-being of their citizens.

The aggressive nature of GC remains a particular challenge to clinical management of this malignancy, and surgical resection of the affected tissues is the only effective treatment, with chemo/radiotherapy providing some benefit as adjuvant treatment. However, the efficacy of GC surgery is reliant upon the disease stage at which it is applied. Delays associated with incorrect or mis-diagnosis of the generally non-specific clinical symptoms in early stage GC (when the tumor is localized and has low risk of metastasis) can completely preclude surgery. Indeed, it has been reported that > 30% of GC patients in China are diagnosed at malignancy stages that are too far advanced for resection to be a feasible (benefit: risk) option[3].

One way to improve timely diagnosis in GC patients is to develop more accurate and sensitive methods of screening. Biomarkers, such as epigenetic modifications, are good candidates for such tests as they are detectable in serum samples and may reflect not only the presence of disease, but also its prognosis (when differential levels correspond to progressive stages of tumor pathology). In addition, diagnostic and prognostic biomarkers represent putative molecular targets of therapeutic strategies and may be exploited to develop more effective, less invasive and more individualized therapies against these aggressive tumors.

Several forms of epigenetic modifications exist, and their various alterations to the chromatin structure affect gene expression and have been implicated in pathological processes underlying a multitude of disease conditions, including tumorigenesis[4,5]. In particular, the post-translational modifications (PTMs) of histones, including acetylation, methylation, phosphorylation and ubiquitination, function as regulators of DNA-associated signaling networks required for normal physiological processes[6], such as cell growth, cycling, and movement - all important features of human cancer[6-9].

Compared to the other histone modifications, ubiquitination is less well studied and its specific roles in many types of tumors remain to be precisely defined. Focused research efforts involving monoubiquitination of lysine 120 on histone H2B (uH2B), however, have begun to elucidate its regulatory mechanism and its downstream effects under normal physiological conditions. Upon catalyzation by ubiquitin-conjugating enzyme (Rad6) and ubiquitin-protein ligase (RNF20)[10,11], uH2B acts to promote or suppress gene transcription[12,13]. Intriguingly, recruitment of RNF20 to gene promoter regions, mediated by transactivators such as Gal4 or p53, has been shown to be required for full induction of transcription of genes related to cancer, such as p21 and MDM2. Furthermore, de-regulation of uH2B has been suggested as an etiology of cancer development[14,15].

The current study was designed to investigate the potential roles of three forms of histone modification, uHB and di- and tri-methylation at H3 lysine 4 (H3K4-2me and H3K4-3me, respectively), in gastric carcinoma and in relation to its clinicopathological features. Detecting cancer type- and stage-specific differential immunohistochemistry (IHC)-staining patterns of histone modifications may represent a useful biomarker-based prognostic method and provide novel insights into potentially manipulable targets of anti-GC molecular therapies.

MATERIALS AND METHODS

Clinical samples

One-hundred-and-fifty-nine formalin-fixed, paraffin-embedded GC tissue specimens obtained from gastrectomy or upper-gastrointestinal endoscopy performed at the Department of Gastrointestinal Surgery and Digestive Endoscopy Center of West China Hospital between January 2006 to January 2007 were selected for analysis. The GC specimens included 23 well-differentiated, 55

moderately-differentiated and 81 poorly-differentiated tumors. According to the Lauren classification system, 60 were intestinal-type and 59 were diffuse-type GC. According to staging by the tumor-node-metastasis (TNM) system, 15 were at stage Ⅰ, 20 were at stage Ⅱ, 99 were at stage Ⅲ and 25 were at stage Ⅳ.

According to the medical records, all GC specimens were obtained during potentially curative surgical resection, and none of the patients had received preoperative chemotherapy or radiotherapy. Follow-up data was available for all patients until December 2012 or until death.

In addition, 20 non-tumor gastric mucosa specimens, including sections from normal and inflammatory epithelium, were randomly selected for use as normal controls.

IHC staining of uH2B, H3K4-2me, and H3K4-3me

The GC and normal control specimens (5 μm) were deparaffinized and incubated with 0.3% hydrogen peroxide in 28% methanol for 30 min to quench the endogenous peroxidase activity. Following EDTA/high-pressure antigen retrieval, the sections were exposed to 1% bovine serum albumin for 20 min to block non-specific binding sites and then to primary antibodies against uH2B, H3K4-2me and H3K4-3me (Cat. No. 05-1312, 05-1338, and 05-1339 respectively; Millipore, Billerica, MA, United States) for 30 min. An additional 15 min post-antibody blocking step was carried out before exposure to the PowerVision+ poly-horseradish peroxidase (HRP)-anti-mouse/rabbit IgG secondary antibodies (Leica Biosystems, Newcastle, United Kingdom) for 30 min and HRP antibody (VECTASTAIN®; Vector Laboratories Inc., Berlingame, CA, United States) for 30 min. Immunoreactivity was visualized upon exposure to the DAB chromogen. The processed tissue sections were then counterstained with hematoxylin, dehydrated and mounted.

Dual-rated semiquantitative analysis of IHC staining levels

The degree of uH2B, H3K4-2me and H3K4-3me immunostaining in each specimen was assessed by two investigators (Yang JL and Wang YP) working independently, as described below. The two sets of results were compared and in the case of disagreement, the section was re-examined by both investigators simultaneously with discussion to achieve a consensus score.

For each processed specimen, three high-power (× 200) magnification fields encompassing an average of 1000 cells (range: 800-1200) were selected (BX51 microscope; Olympus, Tokyo, Japan) and image obtained (FAST 1394 camera with accompanying QCapture suite software; QImaging, Surrey, BC, Canada) to capture an overall representation of different staining densities. An immunoreactivity score (IRS) for each of the three modifications detected was calculated as the product of staining intensity (SI) multiplied by percentage of positively-stained cells (PP)[16]. SI was defined according to a four-point gradient scale, where no staining = 0, weak-coloring (light yellow) = 1, moderate-coloring (bright yellow) = 2, and strong-coloring (brown) = 3. PP was defined according to a four-point positive/negative scale, where 0-9% positive cells = 0, 10%-25% positive cells = 1, 26%-50% positive cells = 2, 51%-75% positive cells = 3, and > 75% positive cells = 4.

The triplicate IRS scores for each of the three detected modifications were averaged for each specimen and used to classify the degree of uH2B, H3K4-2me and H3K4-3me immunostaining as follows: no modification: 0; low-level modification; 1-5; high-level modification: ≥ 6.

Statistical analysis

All statistical analyses were performed by the SPSS software suite, version 13.0 (SPSS Inc., Chicago, IL, United States). The relationships between uH2B modification levels and clinicopathological parameters of GC were examined by a Wilcoxon rank sum test (for pairwise comparisons) and the Kruskal-Wallis H test (for multiple comparisons). The correlation between uH2B modification and survival was estimated by Kaplan-Meier analysis. The role of uH2B as an independent prognostic factor for survival was assessed by multivariate Cox regression analysis. The threshold for statistical significance was set as $P < 0.05$.

RESULTS

uH2B, and not H3K4-2me or H3K4-3me, shows differential IHC staining in GC associated with extent of tumor differentiation

The IHC staining patterns of H3K4-2me and H3K4-3me were similar between the GC and normal control tissues, with the nuclear staining distributed evenly, regardless cancer status or tumor differentiation (Figure 1). In contrast, the uH2B staining patterns and IRS scores were remarkably different between the GC and the normal control tissues, as well as between the different classes of tumor differentiation (Figure 2). All 20 non-tumor mucosa specimens showed high-level uH2B modification (≥ 6 IRS). The amount of GC specimens with high-level uH2B modification decreased in conjunction with increasing level of tumor dedifferentiation, with IRS scores ≥ 6 seen in 65.2% (15/23) of well-differentiated GC tumors, 47.2% (26/55) of moderately-differentiated GC tumors, and 2.4% (2/81) of poorly-differentiated GC tumors. Moreover, this trend of decreased uH2B with increased degree of differentiation was statistically significant ($P < 0.001$, Table 1), suggesting that uH2B may play a role in maintenance of tumor differentiation.

Differential uH2B IHC staining correlates with Lauren classification of the histological type of tumor

When the GC specimens were divided by the Lauren classification, significantly more of the intestinal-type samples showed positive uH2B staining than the diffuse-type samples [90.0% (54/60) *vs* 71.2% (42/59), $P <$

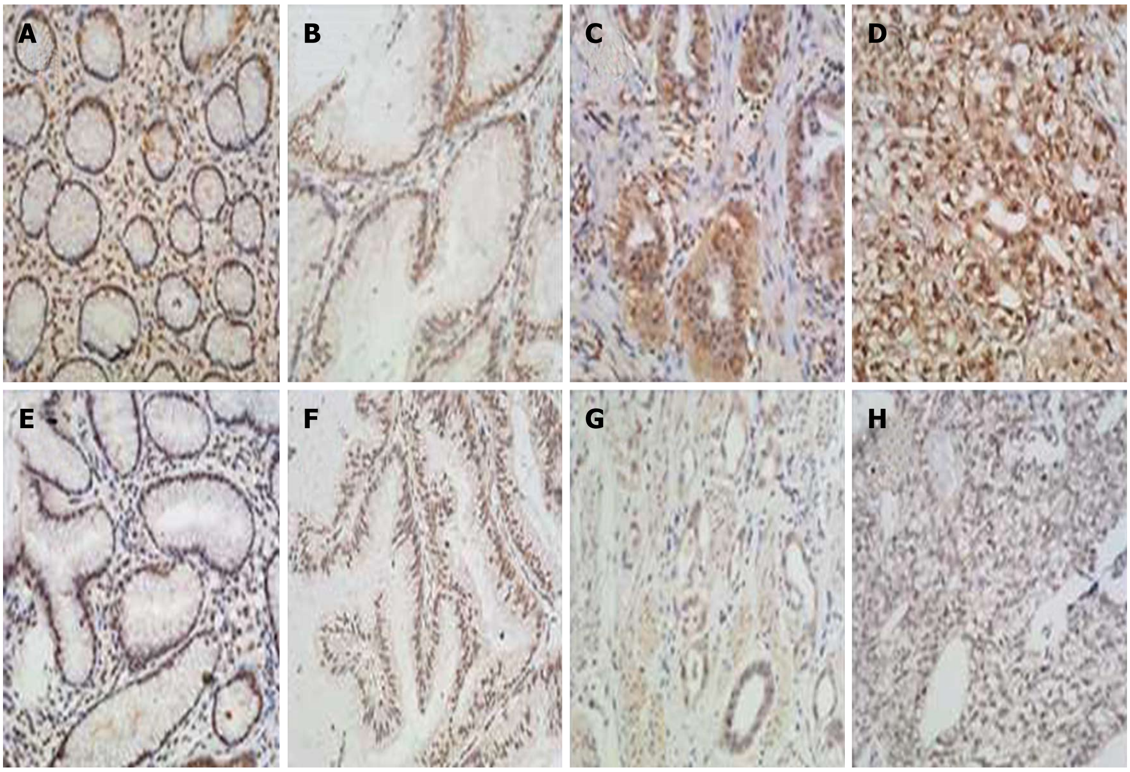

Figure 1 Immunohistochemical nuclear staining of H3K4-2me and H3K4-3me. H3K4-2me (A-D) and H3K4-3me (E-H) in normal gastric mucosa (A, E), well-differentiated gastric cancer (GC) tumor (B, F), moderately-differentiated GC tumor (C, G), and poorly-differentiated GC tumor (D, H). Regardless of the GC differentiation status, H3K4-2me and H3K4-3me displayed high-level nuclear signals, as visualized by immunohistochemistry. Magnification: × 200.

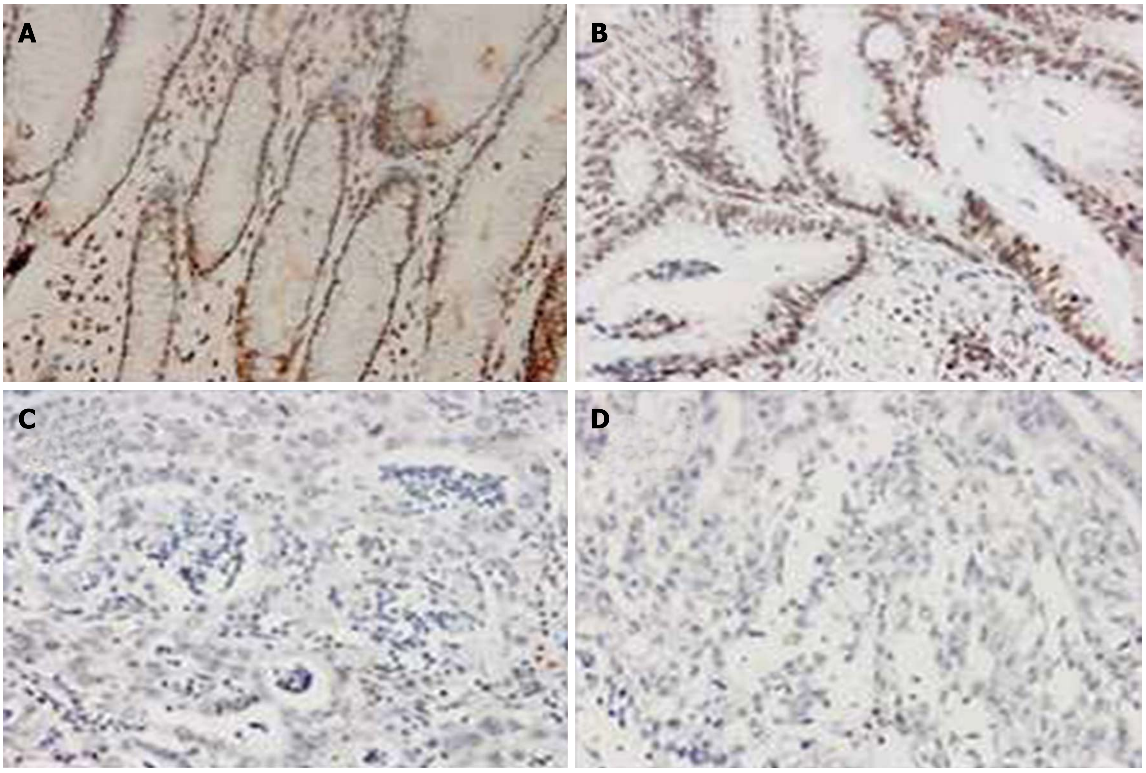

Figure 2 Immunohistochemical detection of staining patterns of uH2B in gastric cancer at various stages of differentiation. A: Normal gastric mucosa shows high-level staining (brown); B: Well-differentiated gastric cancer (GC) shows high-level staining; C: Moderately-differentiated GC shows low-level staining; D: Poorly-differentiated GC shows negative staining. Magnification: × 200.

0.05; Figure 3]. In addition, significantly more of the intestinal-type tumors showed high-level modification

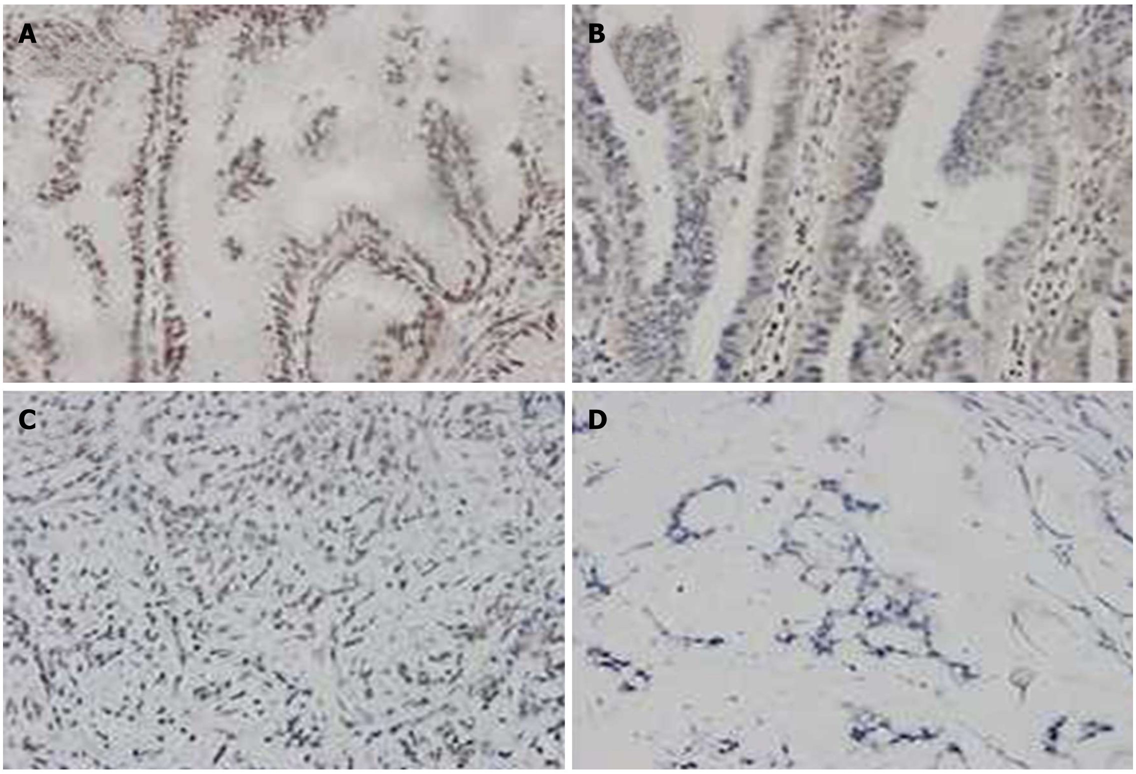

Figure 3 Level of nuclear staining of uH2B according to Lauren classification of tumor type. A, B: Intestinal-type tumors showing (A) high-level staining (brown) of well-differentiated tumors and (B) fewer uH2B+ cells and moderate staining (yellow) of moderately-differentiated tumors; C, D: Diffuse-type tumors showing (C) low-level staining (light yellow) and few uH2B+ cells of poorly-differentiated tumors and (D) negative staining in poorly-differentiated tumors. Magnification: × 200.

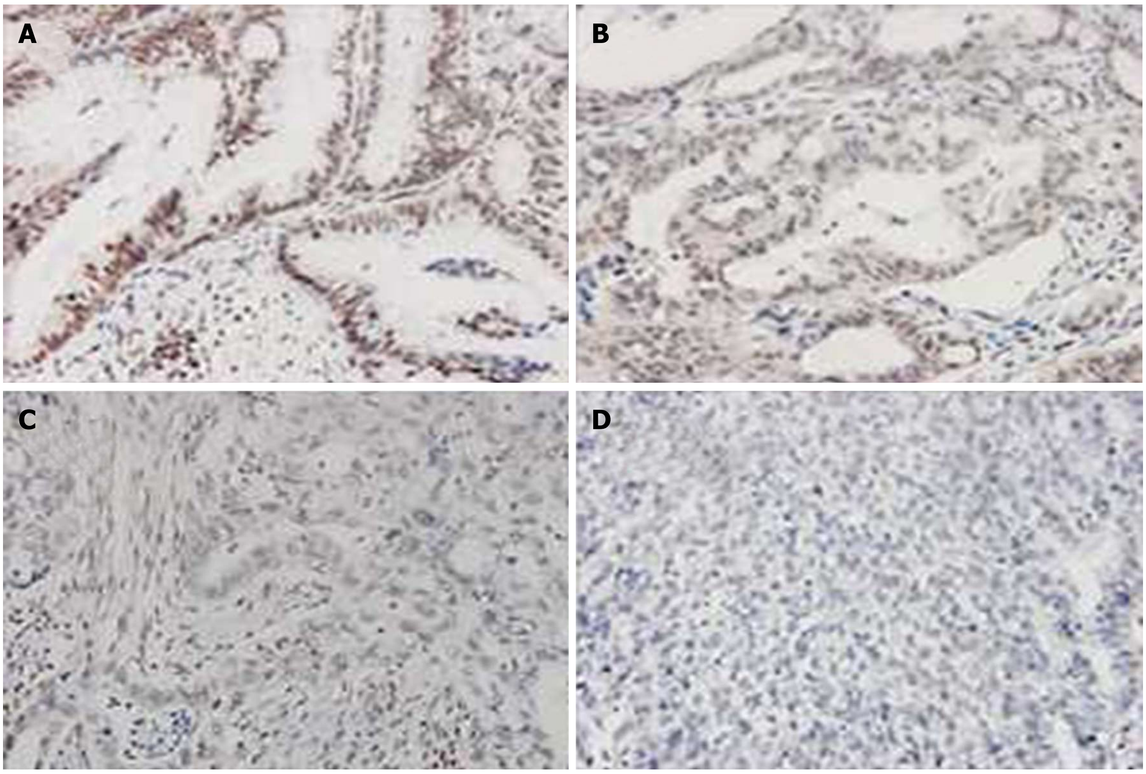

Figure 4 Immunohistochemical detection of uH2B staining at different TNM stages. A: Stage Ⅰ (well-differentiated) gastric cancer (GC) tumor shows high-level staining; B: Stage Ⅱ (moderately-differentiated) GC tumor shows fewer uH2B+ cells and moderate staining (yellow); C: Stage Ⅲ (poorly-differentiated) GC tumor shows few uH2B+ cells and low-level staining; D: Stage Ⅳ (dedifferentiated) GC tumor shows no UH2B+ cells and negative staining. Magnification: × 200.

[55.0% (33/60) *vs* low-level modification: 35.0% (21/60), $P < 0.05$; Figure 3], and this pattern was significantly dif-

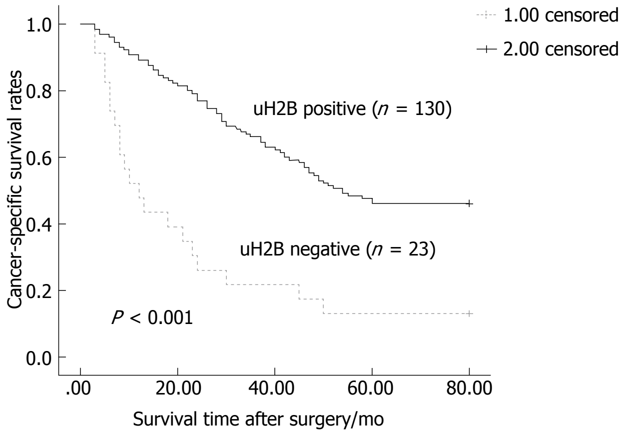

Figure 5 Kaplan-Meier curves of cancer-specific survival for gastric cancer patients based on uH2B+ and uH2B- status, as detected by immunohistochemistry. The 5-year survival rate of patients with positive uH2B staining (*n* = 130) was significantly higher than that of patients with negative uH2B staining (*n* = 23).

Table 1 Immunohistochemical detection of uH2B modification levels and gastric cancer clinicopathological parameters

Parameter	*n*	IHC Staining Level			u/H[1]	*P* value
		Negative	Low	High		
Age (yr)					3059.000	0.969
< 60	93	10	62	21		
≥ 60	66	15	29	22		
Sex					2728.000	0.373
Male	100	13	59	28		
Female	59	12	32	15		
Tumor differentiation					40.376	< 0.001
Well	23	1	7	15		
Moderate	55	7	22	26		
Poor	81	17	62	2		
Lauren classification					933.000	< 0.001
Intestinal	60	6	21	33		
Diffuse	59	17	35	7		
Lymph node metastasis					2330.000	0.049
Absent	53	2	35	16		
Present	106	23	56	27		
TNM stage					12.896	0.005
Ⅰ	15	1	6	8		
Ⅱ	20	2	15	3		
Ⅲ	99	14	55	30		
Ⅳ	25	8	15	2		

[1]u represents the test statistic of the Kruskal-Wallis H test and H represents the Wilcoxon ranks sum test. IHC: Immunohistochemistry.

ferent from that seen in the diffuse-type tumors [*vs* high-level modification in diffuse-type: 11.9% (7/59), *P* < 0.001; Table 1].

Differential uH2B IHC staining correlates with TNM stage and lymph node metastasis

When the GC specimens were divided by TNM stages, a statistically significant trend in differential uH2B modification level was observed. As shown in Figure 4, the frequency of high-level uH2B modification was 53.3% (8/15) in stage Ⅰ tumors, 15.0% (3/20) in stage Ⅱ tumors, 30.3% (30/99) in stage Ⅲ tumors, and 8.0% (2/25) in stage Ⅳ tumors. The difference in frequency of high-level uH2B modification detected in stage Ⅰ and stage Ⅳ tumors reached statistical significance (*P* = 0.005; Table 1).

In addition, GC cases with lymph node metastasis showed a significantly lower frequency of high-level uH2B modification [25.4% (27/106) *vs* no lymph node metastasis: 30.2% (16/53), *P* = 0.049; Table 1].

Table 2 Multivariate analysis for prognostic factors in gastric cancer-specific survival of patients

Variable	Comparison	RR	95% CI	*P* value
Age (yr)	< 60 *vs* ≥ 60	0.761	0.493-1.174	0.217
Sex	male *vs* female	0.961	0.618-1.494	0.858
Tumor differentiation	Well *vs* moderate, poor	0.497	0.301-0.819	0.006
Lymph node metastasis	Present *vs* absent	3.274	1.728-6.201	< 0.001
TNM stage	Ⅰ *vs* Ⅱ, Ⅲ, Ⅳ	1.695	1.112-2.583	0.014
uH2B modification	IHC stain positive *vs* negative	0.400	0.237-0.677	0.001

Prognostic significance of uH2B modification in GC

Of the 159 GC patients treated with surgical resection, 96 (60.4%) died from GC-related causes during the follow-up period and six died from non-GC causes. When the overall patient population was divided by presence of uH2B staining, GC-related deaths were found to have occurred in a significantly higher proportion of patients with negative uH2B staining than those with positive uH2B staining [88.0% (22/25) *vs* 55.2% (74/134), *P* < 0.05]. The cumulative 5-year cancer-specific survival rate was 43.4%. Moreover, the 5-year survival rate of patients with positive uH2B staining was significantly higher than that of patients with negative uH2B staining (52.69 ± 2.45 *vs* 23.74 ± 5.21, *P* < 0.001; Figure 5).

According to Cox multivariate regression, uH2B modification level is an independent prognostic factor for cancer-specific survival of GC patients. The risk of death in patients with negative uH2B staining was 2.5-times (1:0.4) that of patients with positive uH2B modification (RR = 0.40, 95%CI: 0.237-0.677, *P* = 0.001; Table 2).

DISCUSSION

In this study, immunohistochemical detection of human GC samples was performed as a semi-quantitative approach to measure the H2B monoubiquitination at lysine 121 and investigate its potential clinical significance with regards to diagnosis (GC *vs* control tissues) and prognosis (progressive stages of GC tumorigenesis). To the best of our knowledge, this study provides the first evidence of correlation between uH2B modification level and clinicopathological and prognostic features of human GC, including tumor differentiation, Lauren's classification, lymph node metastasis, and TNM stage but not with sex or age (data not shown).

The modification of H2B at lysine 121, shown by

the percentage of positivity and intensity of immunohistochemical detection, was significantly less robust in tumors of lower differentiation level. The degree of differentiation is considered to be strongly associated with the malignancy of cancer; therefore, this result indicates that reduced uH2B may be correlated with a worse prognosis. The more frequent and intense staining of uH2B observed in intestinal-type tumors in the current study, compared to the diffuse-type tumors, suggests that loss of uH2B may contribute to GC tumor progression. From a histological perspective, the composition of intestinal-type GC tumors includes a remarkable amount of ductal structures, displaying a better differentiation than the diffuse-type GC tumors that may be related to the better prognosis of the former tumor type[17,18]. Finally, the current observation of lower uH2B modification level in higher grade TNM stages, which tend to be more aggressive and invasive towards the inner tissues and more metastatic, suggest that this modification may be a useful predictive biomarker of the invasive potential of a GC specimen.

The collected results of the current study indicate that the progressive stages of GC are accompanied by differential uH2B modification levels that are detectable by IHC. Prenzel *et al*[19] reported a similar finding for human specimens of breast cancer. Specifically, the abundant uH2B signals detected by IHC in normal mammary epithelium and benign breast tumors were absent in most malignant and metastatic breast tumors. Urasaki *et al*[20] also demonstrated drastically reduced uH2B modification levels in breast, colon and lung cancer cells, as compared to the abundant expression in matched normal control tissues. Thus, loss of uH2B may lead to progression and metastasis of tumors, in general.

The mechanisms underlying tumor-related decreases in uH2B remain unknown. Besides the known Rad6 and RNF20 regulatory enzymes[10,11], other de/ubiquitinating enzymes are likely to be involved in the dynamic process of uH2B promotion of tumorigenesis. For example, the ubiquitin-specific protease 22 (USP22), a member of the recently identified polycomb/cancer stem cell signature[21], has been shown to deubiquitinate H2Aub1 or H2Bub1 *in vitro*, suggesting functions in epigenetic regulation, cancer progression and transcription activation[22,23]. If USP22 plays a role in the cancer-related differential uH2B modifications, then corresponding changes in USP22 expression may be detected.

In fact, studies of USP22 expression level in cancer have demonstrated significant upregulation in malignant tumors (compared to the normal low or moderate levels in non-cancerous skeletal, heart, muscle, liver and lung tissues)[24] and significant correlations to tumor relapse, invasion depth, pathological stage and lymph node metastasis[25]. Moreover, a study of primary GC showed that upregulated USP22 protein expression was related to lymph node metastasis[26]. Future investigations may elucidate a role for USP22 in the de-regulation of uH2B, particularly in GC.

Another potential regulator of tumor-related decreases in uH2B is the ubiquitin-specific peptidase 49 (USP49) complex, which specifically deubiquitinates histone H2B *in vivo* to enhance the stability of a nucleosome spanning the affected exons[27]. Again, further research is required to determine whether USP49 acts as a histone H2B-specific deubiquitinase to promote tumorigenesis.

While the current study provided clear evidence of decreased uH2B in malignant and poorly-differentiated human GC specimens, the physiological significance of the loss of this histone code remains unclear. The critical roles of H3K4-2me and H3K4-3me modifications in gene expression and cellular viability are well established; however, the requirement for uH2B in these processes remains controversial. Genome-wide studies have shown that uH2B is associated mostly with actively transcribed genes in mammals, suggesting that H2B is universally required for gene transcription. However, loss of the H2B-specific RNF20/40 enzyme produced only moderate effects on a fraction of the transcriptome and no overall effects on cell viability[28]. This apparent differential function of H3K4 modifications and the uH2B modification is in line with the observations of the current study of GC tissues, whereby the uH2B level showed a unique gradual decrease from the benign to malignant stages.

In conclusion, the compelling evidence of uH2B not being required for viability of malignant cells in gastric carcinoma provided by the current study indicates that the decrease of uH2B might be an early event in GC and could be a counteracting factor against carcinogenesis of GC.

COMMENTS

Background

Gastric cancer (GC) remains a significant healthcare burden worldwide, with high mortality rates in countries with the highest incidences, such as China. Surgery is currently the only effective treatment, but must be applied in the early stages when the tumor is less aggressive. Unfortunately, the asymptomatic nature of early stage GC leads to missed diagnosis and precludes early surgical management. There is an urgent need to identify sensitive biomarkers that accurately diagnose GC at the early stage, as well as indicate prognosis for a particular patient. Such factors may also represent novel targets of molecular therapies, helping to overcome the limitations and risks associated with surgical resection.

Research frontiers

The physiological and pathological roles of monoubiquitination on lysine 120 of histone H2B (uH2B) remain to be fully elucidated. As a general transcriptional regulator, de-regulation of uH2B may contribute to tumorigenesis and cancer progression by promoting expression of cancer-associated genes or suppressing expression of anti-tumor genes. Investigating the differential level of uH2B in specific cancer types, such as GC, will provide insights into its clinicopathological and prognostic significance.

Innovations and breakthroughs

In this study, immunohistochemical (IHC) analysis of human GC specimens was used to detect the tumor-related levels of uH2B modification. Application of a dual-rated semi-quantitative method to score the IHC results allowed for statistical correlation analysis of uH2B modification level and tumor-related features. The GC-related decrease in uH2B modification level was positively correlated with extent of tumor dedifferentiation, intestinal-type tumors, occurrence of lymph node metastasis, worse TNM stage and lower 5-year survival rate. IHC-detected differential uH2B

modification may be developed as clinically useful prognostic biomarker in early GC.

Applications

The uH2B differential staining pattern detected by IHC that accompanies progressive stages of GC not only indicated a potentially important role for this histone modification in carcinogenesis, but also suggested its potential as a target of molecular therapy.

Terminology

uH2B, the monoubiquitination on lysine 120 of histone H2B, is a general transcriptional regulator, and its deregulation has been implicated in various cancers. IHC is a well-established laboratory detection method that exploits the antibody-antigen binding reaction to identify and visualize the location and level of a target protein in tissues or individual cells.

Peer review

This paper is an interesting article regarding the role of histone modification events in the development of GC. The results of the paper show that decrease of uH2B might be an early event in GC, which could be a counteracting factor against carcinogenesis of gastric cancer. Furthermore, histone modification plays important roles in understanding the pathogenesis of gastric carcinoma, and could be a potential therapeutic target in the future

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BRIEF ARTICLE

siRNA-targeted inhibition of growth hormone receptor in human colon cancer SW480 cells

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Abstract

AIM: To determine the effects of RNAi-mediated inhibition of the growth hormone receptor (*GHR*) gene on tumors and colon cancer cells *in vivo*.

METHODS: Construction of a eukaryotic vector for human GHR expression, the pcDNA™6.2-GW/EmGFP-small interfering RNAs (siRNAs)-GHR plasmid, was used to inhibit GHR expression. Thirty-six BALB/c nude mice were randomly divided into groups and treated with normal saline (NS), recombinant plasmid (G_2), growth hormone (GH), 5-fluorouracil (FU), G_2+FU or G_2+FU+GH. Each nude mouse was subcutaneously inoculated with 1×10^7 human colon cancer SW480 cells; the nude mice were weighed before inoculation and on the 2nd, 5th, 8th, 11th, 14th and 17th day after inoculation. All nude mice were sacrificed after 17 d. Each subcutaneous tumor was removed and studied. Tumor volume was measured on the 5th, 8th, 11th, 14th and 17th day after inoculation. The expression of GHR protein in the tumor tissue was detected by Western blotting analysis, and the differences in GHR mRNA expression in the tumor tissue were detected by real-time quantitative reverse transcription-polymerase chain reaction.

RESULTS: Compared to the control group, the weights of the inoculated nude mice on the 17th day after inoculation were: G_2: 21.60 ± 0.71 g, GH: 21.64 ± 0.45 g, FU: 18.94 ± 0.47 g, FU+G_2: 19.40 ± 0.60 g, G_2+FU+GH: 21.04 ± 0.78 g *vs* NS: 20.68 ± 0.66 g, $P < 0.05$; the tumor volumes after the subcutaneous inoculation were: G_2: 9.71 ± 3.82 mm^3, FU: 11.54 ± 2.42 mm^3, FU+G_2: 11.42 ± 1.11 mm^3, G_2+FU+GH: 10.47 ± 1.02 mm^3 *vs* NS: 116.81 ± 10.61 mm^3, $P < 0.05$. Compared to the GH group, the tumor volumes were significantly decreased in the experimental groups. The GHR protein expression (G_2: 0.39 ± 0.02, FU: 0.40 ± 0.02, FU+G_2: 0.38 ± 0.01, G_2+FU+GH: 0.39 ± 0.01 *vs* NS: 0.94 ± 0.02, $P < 0.05$) and the GHR mRNA expression (G_2: 14.12 ± 0.10, FU: 15.15 ± 0.44, FU+G_2: 16.46 ± 0.27, G_2+FU+GH: 15.37 ± 0.57 *vs* NS: 12.63 ± 0.14, $P < 0.05$) were significantly decreased and increased, respectively, in the experimental groups.

CONCLUSION: Inhibition of GHR in human colon cancer SW480 cells resulted in anti-tumor effects in nude mice.

Key words: Growth hormone receptor; Small interfering RNAs; Colon cancer; Gene therapy; Signaling pathway

Core tip: Human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. GH/GHR plays an important role in colon cancer emergence and development. After specific binding of GH to GHR in tumor tissues, the JAK-STAT signaling pathway is activated, resulting in improved cell growth and proliferation. small interfering RNAs (siRNAs)-targeted inhibition of the human *GHR* gene was used to investigate

its impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to GHR suppression. The siRNA-containing plasmid could suppress GHR expression in colon cancer cells and exhibited anti-tumor effects in nude mice.

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INTRODUCTION

As shown previously by our team, human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. Additionally, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR demonstrates an upward tendency[1,2]. Some researchers[3-6] believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor and that GH and GHR play important roles in the emergence and development of colon cancer. After specific binding of GH to GHR in tumor tissues, the JAK-STAT signal transduction pathway is activated, resulting in improved cell growth and proliferation[7-10]. Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways[11-13].

In this study, siRNA targeting the human *GHR* gene was used to investigate the impact that GHR has on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of GHR expression.

MATERIALS AND METHODS

Experimental animals and cell lines

Thirty-six 8-wk old, female BALB/c nude mice, weighing between 20 and 22 g, were purchased from Vital River Laboratories (VRL) with license No. SCXK (Jing) 2006-0009. The mice were kept in the SPF environment of the animal experiment center in Kunming Medical University. The human colon cancer cell line SW480 was obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science.

Laboratory reagents

The HQ high purity plasmid extraction kit was purchased from Invitrogen (Invitrogen, Carlsbad city, California, United States). The BCA protein concentration kit (Tiangen Biology and Chemistry) and the molecular mass albumin standard were purchased from Tiangen Biology and Chemistry (Fermentas Company). The mouse monoclonal anti-human GHR antibody was obtained from R and D Company (MAB1210), and the goat secondary anti-mouse IgG-HRP antibody was purchased from Abmart Company. RNase H was obtained from Invitrogen, and the Golden Taq PCR kit was purchased from Tiangen Biology and Chemistry. SYBR Green-Real Master Mix was purchased from Tiangen Biology and Chemistry, and all primers used in the study were obtained from Invitrogen.

Preparation of cell suspension

Colon cancer SW480 cells were cultivated in RPMI 1640 nutrient solution supplemented with 10% fetal calf serum (FCS), 10.0 × 10^3 U/L penicillin, and 100 mg/L streptomycin in a 37 ℃ incubator with 50 mL/L CO_2. This is an adherent cell line. Cells in the exponential growth phase were harvested using 0.25% trypsin, and the cells were resuspended using a machine. The cells were then centrifuged at 2000 rpm for 5 min. Then, the supernatant was removed, and the cells were resuspended in physiological saline at a concentration of 1 × 10^7 cells/mL. Trypan blue staining was used to ensure that cell viability was above 95%; after resuspension the cells were stored in an ice bath.

Construction of siRNA and eukaryotic expression vectors

siRNA oligonucleotides were designed against the mRNA sequence of human *GHR* found in GenBank, which had a total length of 4414 bp (Accession No.: X06562, GI: 31737). We used the RNAi Designer website (http://bioinfo.clontech.com/rnaidesigner) to design the siRNA that targeted the hGHR mRNA (527-547: GTCAGTTTAACTGGGATTCAT). Using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search the EST database, we found that the siRNA was not homologous to another gene and would be an effective siRNA sequence. The complementary single strand primer was incubated at 94 ℃ in annealing buffer solution for 3 min, and the oligonucleotides were annealed at 37 ℃ for 1 h. The annealed oligonucleotides were then phosphorylated at 37 ℃ for 30 min with T4 DNA-PNK. The oligonucleotides were then ligated into the linearized (BamHI/HindIII) pcDNA™ 6.2-GW/EmGFP-GHR-siRNA plasmid using T4 DNA ligase. The final product was transformed in competent *E. coli* DH5α cells, and the transformants were spread on transformation plates containing 50 μg/mL spectinomycin dihydrochloride (Sigma, Catalog No. S4014). The plates weres kept in a 37 ℃ incubator overnight, and three coenobium clones were picked from each plate and subcultured. The plasmids were extracted using plasmid extraction kits, and the plasmids were confirmed by restriction enzyme digestion with *EcoR* Ⅰ, *Sac* Ⅰ and *Sal* Ⅰ. The transformation liquid was also sequenced to ensure that recombination had not occurred in the insert fragments during the cloning process. Finally, the pcDNA™6.2-GW/EmGFP-GHR-siRNA plasmids were constructed successfully and are referred to as G_2 throughout the paper. Additionally, the plasmids were extracted and diluted in

DMEM as a precaution.

Groups and method of drug distribution

We subcutaneously injected 1 × 10^7 human colon cancer SW480 cells into BALB/c nude mice. On the first day after the injection, the mice were divided into six groups and administered the indicated drug and dose. (1) Normal saline (NS): 10 μL of NS was injected into the abdominal cavity of each mouse; (2) Plasmid (G_2): 10 μg of the G_2 eukaryotic expression plasmid was injected subcutaneously into each mouse; (3) Growth hormone (GH): 2 IU/kg rhGH, a physiological dose of GH, was hypodermically injected into each mouse; (4) 5-fluorouracil (FU): 20 mg/kg 5-FU was injected into the abdominal cavity of each mouse; (5) 5-FU+plasmid (FU+G_2): 20 mg/kg 5-FU and 10 μg of the G_2 plasmid were injected into the abdominal cavity of each mouse; (6) 5-FU+GH+plasmid (FU+GH+G_2): 20 mg/kg 5-FU, 10 μg of the G_2 plasmid, and 2 IU/kg rhGH were injected into the abdominal cavity of each mouse; and (7) Each of the above groups was treated every 5 d for three rounds of treatment.

Observation index

Observation of weight and tumor volume of nude mice: The weight of the nude mice was recorded before injection of the SW480 cells and on the 2^{nd}, 5^{th}, 8^{th}, 11^{th}, 14^{th} and 17^{th} day after injection. The length and the minimum diameter of the tumors were recorded, and the tumor volume was calculated on the 5^{th}, 8^{th}, 11^{th}, 14^{th} and 17^{th} day after hypodermic injection of human colon cancer SW480 cells The following equation was used to calculated the tumor volume: $V = (A \times B^2)/2$, where A represents the major diameter and B represents the minimum diameter.

Expression of GHR in tumor tissues as detected by Western blotting analysis

A sample of each tumor was removed from the nude mice and cut into pieces. Cleanser lysate solution containing PMSF (400 μL) was added to the tumor sample in a homogenizer. After the cells were lysed for 30 min, the homogenate was centrifuged at 12000 rpm for 5 min at 4 ℃. The supernatant was removed, placed in 0.5 mL centrifuge tubes, and stored at -20 ℃. Then, 20 μL of the lysate sample was separated and analyzed using SDS-PAGE; the proteins were electrotransferred onto nitrocellulose membranes and detected using a chemiluminescent detection system. Beta-actin was used as a loading control. The images were analyzed using the BandScan5.0 program. The ratio of GHR expression to beta-actin expression was analyzed using the integral optical density value (RV) of the band in the same sample. The results are shown as the expression of GHR relative to beta-actin, and the results were measured as, mean ± SD.

Expression of GHR mRNA in tumor tissue was detected by quantitative reverse transcription-polymerase chain reaction

Twenty microliters of the lysate sample was centrifuged at 5000 rpm for 10 min. Prechilled PBS was used to wash the cells, and total RNA was extracted using the Trizol reagent in a one-step method. For first strand synthesis, 1 μg of total RNA was combined with 1 μL of 0.5 μg/μL oligo primer and 12 μL of deionized water. The mixture was then incubated at 70 ℃ for 5 min. Then, the samples were quenched in a bath of ice water and centrifuged at 5000 rpm for 4 s. Next, 4 μL of 5 × reaction buffer, 1 μL of 20 U/μL ribonuclease, and 2 μL of 10 μmol/μL dNTPs were added, and the mixture was incubated at 37 ℃ for 5 min. Then, 1 μL of 200 U/μL reverse transcriptase was added, and the reaction was incubated at 42 ℃ for 60 min, followed by a 10 min incubation at 70 ℃. After the reaction, the cDNA was incubated at 0 ℃ and was stored at -20 ℃. Real time PCR with the SYBR-Green fluorochrome was used to detect the expression of GHR mRNA. For this reaction, 10 μL of cDNA, 2 μL of both forward and reverse primers, 10 μL of buffer solution, 4 μL of ddH_2O, and 1 μL of the ROX fluorochrome were incubated at 95 ℃ for 10 min, followed by 35 cycles of 94 ℃ for 10 s, 56 ℃ for 30 s, and 72 ℃ for 30 s; a final extension time of 5 min at 72 ℃ ended the reaction. GAPDH was used as a negative control. The following primers were used in this experiment: GHR forward: GCAGCTATCCTTAGCAGAGCAC; GHR reverse: AAGTCTCTCGCTCAGGTGAACG; GAPDH forward: GGTCTCCTCTGACTTCAACA; and GAPDH reverse: GAGGGTCTCTCTCTTCCT. The levels of GHR mRNA in the transfection group and the control group were determined by quantitative PCR, and the △CT value of the GHR mRNA was determined between the two groups. It was demonstrated that a higher △CT value represented a larger inhibition of GHR mRNA.

Statistical analysis

Data were expressed as the mean ± SD. Univariate or multivariate data were analyzed using variance analysis and pairwise comparison *q* test by SPSS 18.0 statistical software package. Statistical significance was considered at $P \leqslant 0.05$.

RESULTS

Weight changes in the tumor-bearing mice

At the end of the experiment, all 36 tumor-bearing mice survived from inoculation 2 to 17 d, except for the NS group. After injection, there was a statistically significant difference ($P < 0.05$) in the weight of the mice compared with their weight before injection. The weight of the GH group increased after inoculation with SW480 cells, while the other groups decreased in weight ($P < 0.05$). The weight of the G_2, FU, and G_2+FU groups noticeably decreased ($P < 0.05$) compared with the NS group. The weight of the FU and G_2+FU groups decreased compared with the G_2 group; however, this change was not significant. After the addition of GH, the weight of the G_2+GH+FU group increased compared with the FU group in the same period ($P < 0.05$; Table 1).

Table 1 Weight changes of tumor-bearing mice (n = 6; mean ± SD)

Time	Weight (g, mean ± SD)					
	NS	G_2	GH	FU	FU + G_2	G_2 + FU + GH
Pre-operation	20.69 ± 0.67	21.92 ± 0.70	20.67 ± 0.57	21.93 ± 0.58	21.86 ± 0.73	21.25 ± 0.79
Inoculation 2 d	20.64 ± 0.60	21.86 ± 0.79	20.82 ± 0.56	20.89 ± 0.66[1]	20.09 ± 0.55[1,3]	20.41 ± 0.85[1,3]
Inoculation 5 d	20.65 ± 0.49	21.56 ± 0.81	20.96 ± 0.54[1]	20.94 ± 0.67[1]	19.92 ± 0.58[1,3,5]	20.42 ± 0.76[1]
Inoculation 8 d	20.65 ± 0.65	21.53 ± 0.56[1]	21.18 ± 0.44[1]	20.41 ± 0.73[1,3]	19.92 ± 0.52[1,2,3,5]	20.53 ± 0.70[1,3]
Inoculation 11 d	20.67 ± 0.63	21.53 ± 0.73[1]	21.27 ± 0.53[1,4]	19.86 ± 0.57[1,3]	20.06 ± 0.52[1,2,3,5]	20.63 ± 0.81[1,5]
Inoculation 14 d	20.61 ± 0.62	21.60 ± 0.68[1,2]	21.51 ± 0.44[1,4]	9.46 ± 0.52[1,2,3]	19.86 ± 0.92[1,2]	20.86 ± 0.72[1,4]
Inoculation 17 d	20.68 ± 0.66	21.60 ± 0.71[1]	21.64 ± 0.45[1,2,4]	18.94 ± 0.47[1,2,3]	19.40 ± 0.60[1,2,3]	21.04 ± 0.78[1,4]

[1]Compared with pre-operation, P < 0.05; [2]Compared with NS group, P < 0.05; [3]Compared with G_2 group, P < 0.05; [4]Compared with FU group, P < 0.05; [5]Compared with GH group, P < 0.05. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

Table 2 Tumor volume changes in tumor-bearing mice (n = 6, mean ± SD)

Time	Subcutaneous tumor volume (mm^3, mean ± SD)					
	NS	G_2	GH	FU	FU + G_2	G_2 + FU + GH
Inoculation 5 d	7.72 ± 1.61	7.93 ± 1.74	8.11 ± 1.65	7.42 ± 1.51	6.51 ± 1.20	7.33 ± 1.32
Inoculation 8 d	20.19 ± 4.91[2,3]	13.44 ± 4.12[1,3]	33.28 ± 3.24[1,2,3]	17.51 ± 5.75[3]	15.12 ± 5.01[3]	15.44 ± 4.23
Inoculation 11 d	106.02 ± 6.61[2,3]	21.12 ± 4.04[1,3]	151.90 ± 8.31[1,2,3]	21.00 ± 5.07[1,3]	19.22 ± 4.33[1,3]	22.97 ± 4.95[1,2,3]
Inoculation 14 d	133.41 ± 6.43[2,3]	20.00 ± 4.75[1,3]	178.93 ± 3.11[1,2,3]	16.23 ± 6.51[1,3]	11.55 ± 4.11[1,2,3]	12.12 ± 3.11[1,2,3]
Inoculation 17 d	116.81 ± 0.61[2,3]	9.71 ± 3.82[1,3]	149.01 ± 3.02[1,2,3]	11.54 ± 2.42[1,3]	11.42 ± 1.11[1,3]	10.47 ± 1.02[1,3]

[1]Compared with NS group, P < 0.05; [2]Compared with G_2 group, P < 0.05; [3]Compared with GH group, P < 0.05. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

Changes in tumor volume in the tumor-bearing mice of all groups

By the end of the experiment, the tumor volume of the mice in all groups increased compared to the fifth day after inoculation. The tumor volume of the GH group had the most dramatic increase, followed by the NS group. The tumor volumes of the G_2, FU, G_2+FU, and G_2+GH+FU groups only slightly increased. Compared with the NS group in the same period, the tumor volume of the experimental group obviously decreased, whereas that of the GH group significantly increased (P < 0.05). Compared with the G_2 group, the G_2+FU group had a more pronounced tumor inhibition (P < 0.05). There was no obvious difference in the tumor volume of the G_2+GH+FU group compared with the G_2, FU, and G_2+FU groups in the same period (Table 2).

GHR protein expression in the subcutaneous tumors of tumor-bearing mice in all groups

The expression levels of GHR protein in the tumors of the GH (0.94 ± 0.02) and NS (0.94 ± 0.02) mice were significantly higher than the GHR levels in the G_2 (0.39 ± 0.021), FU (0.40 ± 0.02), G_2+FU (0.38 ± 0.01) and G_2+FU+GH (0.39 ± 0.01) mice. However, there was no significant difference between the G_2 group and the FU, G_2+FU, and G_2+FU+GH groups (P > 0.05).

GHR mRNA expression in the subcutaneous tumors of tumor-bearing mice

Compared with the NS group and the control group that did not have a plasmid, the △CT value of the G_2, FU, G_2+FU, and G_2+FU+GH groups significantly increased (P < 0.05). In the experimental groups, the inhibition ratios of the FU+G_2 and FU+G_2+GH groups against GHR mRNA were higher than that of the G_2 group (P < 0.05; Table 3).

DISCUSSION

Colon cancer is one of the most common malignant tumors[14-16]. Currently, the treatment for colon cancer is surgery combined with radiotherapy and chemotherapy. However, most patients cannot undergo operation or do not respond to chemotherapeutics, leading to the failure of the therapy. Determining the appropriate tumor target spot related to gene and specific therapy has become a hotspot of research in tumor therapy[17]. Due to its action as an anabolic agent and mitogen, GH has a wide range of functions in substance metabolism and body fluid equilibrium, which can accelerate the use of nitrogen and improve the synthesis of liver and muscle proteins. The nutritional effect of GH has already been shown in cachexia[18]. Because GH can increase the brittleness of chromosomes, which, in turn, can cause malignant transformation of cells, and can increase tumor growth, GH has been excluded as a therapy option for the treatment of tumors[19,20]. It has been demonstrated by many epidemiological researchers that patients who receive long term treatment with growth hormone have an increased risk of colon cancer[3,21], and GHR expression in the colon might relate to the occurrence, development and metastasis of these tumors[1,22]. The presence of GHR in the local tissue is a prerequisite for GH to play its role, which means that when determining

Table 3 △CT value of growth hormone receptor mRNA expression detected by real-time reverse transcription-polymerase chain reaction in all groups

	NS	LP	Negative	GH	G_2	FU	FU + G_2	G_2 + FU + GH
△CT	12.63 ± 0.14	12.63 ± 0.43	12.67 ± 0.21	12.71 ± 0.39	14.12 ± 0.10[1]	15.15 ± 0.44[1]	16.46 ± 0.27[1,2]	15.37 ± 0.57[1,2]

[1]Compared with NS group, P < 0.05; [2]Compared with G_2 group, P < 0.05. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

whether to use GH as a therapy, it is important to know the expression and distribution of GHR in a specific tumor cell. GHR is highly expressed in colon cancer tissues[1,2], and high GHR expression has been correlated with poor patient prognosis[23].

RNAi refers to complementary double-stranded RNAs (dsRNAs) that bind to specific endogenous mRNAs, resulting in the degradation of those mRNAs and the silencing of gene expression. Aiming at the relevant signal of the auxanodifferentiation of tumor cells for transduction and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of a tumor specifically and highly efficiently[24,25].

In this research, we constructed plasmids containing siRNAs that targeted the expression of GHR in colon cancer cells, thereby decreasing the expression of GHR in the colon cancer tissues and blocking the GHR-induced signal transduction that promotes tumor cell growth. The results demonstrated that, compared to the control group, the tumor volume and the mRNA and protein expression of GHR in the tumor tissue significantly decreased. Additionally, the combination of GHR silencing and 5-FU treatment had an anti-tumor effect. After the siRNA blocked the expression of GHR in the tumor tissue, the addition of GH could bind to the GHR in the normal tissue. As a result, the weight and nutrition of the nude mice may improve, and GH treatment increases the nude mouse tolerance towards chemotherapy and increases the chemosensitivity of the tumor cells[9]. Compared with the other experimental groups, the FU+G_2+GH group had no significant difference in its reduced tumor volume or decreased expression of GHR protein and mRNA.

Our research showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

COMMENTS

Background

Human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. In addition, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR increases. Some researchers believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor, and GH/GHR plays an important role in the emergence and development of colon cancer. After specific binding between GH and GHR in tumor tissue, the JAK-STAT signal transduction pathway is activated, leading to improved cell growth and proliferation.

Research frontiers

Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways. RNAi refers to complementary double-stranded RNAs that bind endogenous mRNAs, resulting in the specific degradation of that mRNA, which leads to decreased expression of that gene. Aiming at the relevant signal of the auxanodifferentiation of tumor cells and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of tumor specifically and highly efficiently.

Innovations and breakthroughs

In this study, siRNA-targeted inhibition of the *GHR* gene was used to investigate GHR's impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of *GHR* gene expression.

Applications

The results showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

Peer review

The manuscript is well designed and had appropriate methodology.

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META-ANALYSIS

Laparoscopic *vs* open total gastrectomy for gastric cancer: A meta-analysis

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Abstract

AIM: To conduct a meta-analysis comparing laparoscopic total gastrectomy (LTG) with open total gastrectomy (OTG) for the treatment of gastric cancer.

METHODS: Major databases such as Medline (PubMed), Embase, Academic Search Premier (EBSCO), Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library were searched for studies comparing LTG and OTG from January 1994 to May 2013. Evaluated endpoints were operative, postoperative and oncological outcomes. Operative outcomes included operative time and intraoperative blood loss. Postoperative recovery included time to first flatus, time to first oral intake, hospital stay and analgesics use. Postoperative complications comprised morbidity, anastomotic leakage, anastomotic stenosis, ileus, bleeding, abdominal abscess, wound problems and mortality. Oncological outcomes included positive resection margins, number of retrieved lymph nodes, and proximal and distal resection margins. The pooled effect was calculated using either a fixed effects or a random effects model.

RESULTS: Fifteen non-randomized comparative studies with 2022 patients were included (LTG - 811, OTG - 1211). Both groups had similar short-term oncological outcomes, analgesic use (WMD -0.09; 95%CI: -2.39-2.20; P = 0.94) and mortality (OR = 0.74; 95%CI: 0.24-2.31; P = 0.61). However, LTG was associated with a lower intraoperative blood loss (WMD -201.19 mL; 95%CI: -296.50--105.87 mL; P < 0.0001) and overall complication rate (OR = 0.73; 95%CI: 0.57-0.92; P = 0.009); fewer wound-related complications (OR = 0.39; 95%CI: 0.21-0.72; P = 0.002); a quicker recovery of gastrointestinal motility with shorter time to first flatus (WMD -0.82; 95%CI: -1.18--0.45; P < 0.0001) and oral intake (WMD -1.30; 95%CI: -1.84--0.75; P < 0.00001); and a shorter hospital stay (WMD -3.55; 95%CI: -5.13--1.96; P < 0.0001), albeit with a longer operation time (WMD 48.25 min; 95%CI: 31.15-65.35; P < 0.00001), as compared with OTG.

CONCLUSION: LTG is safe and effective, and may offer some advantages over OTG in the treatment of gastric cancer.

Key words: Gastric cancer; Laparoscopic total gastrectomy; Laparoscopic assisted total gastrectomy; Open

total gastrectomy; Meta-analysis

Core tip: Currently, surgical resection is the mainstay treatment for gastric cancer. With technical advances and improved instrumentation, laparoscopic total gastrectomy (LTG) is being used increasingly to treat this malignant disease. However, compared with conventional open total gastrectomy (OTG), the safety and technical feasibility of LTG have not been adequately evaluated. This study clarified that, compared with OTG, LTG has similar short-term oncological outcomes, analgesic use and mortality. Furthermore, LTG was associated with lower intraoperative blood loss and overall complication rate, fewer wound-related complications, quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operation time.

Xiong JJ, Nunes QM, Huang W, Tan CL, Ke NW, Xie SM, Ran X, Zhang H, Chen YH, Liu XB. Laparoscopic *vs* open total gastrectomy for gastric cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(44): 8114-8132 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8114.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8114

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide and is a leading cause of cancer death[1]. Despite improvements in diagnosis and systemic therapy, surgery, in the form of gastrectomy with lymph node dissection, still forms the mainstay of treatment[2]. Since it was first described in 1994[3], laparoscopic surgery, and more specifically laparoscopic distal gastrectomy, has been used widely in the far East to treat early gastric cancers and is associated with many advantages over open surgery[4-7]. On the other hand, laparoscopic total gastrectomy (LTG) with lymph node dissection, which was reported in 1999[8], is practiced less widely and is more challenging to perform[9]. The procedure is associated with a high risk of bleeding and a technically demanding anastomosis, all within a narrow operating field[9,10]. However, with technical advances and improved instrumentation, LTG is now being used increasingly to treat gastric cancer[11-14].

A number of studies comparing the short-term or long-term outcomes, of LTG *vs* conventional open total gastrectomy (OTG) for early and advanced gastric carcinoma have shown it to be feasible, oncologically effective and safe in experienced hands[14-17]. LTG offers the potential advantage of being less invasive, causing less surgical trauma with less postoperative pain and a quicker recovery[18,19]. However, most studies were too small to adequately evaluate the surgical outcomes of LTG. The aim of the current study was to inform future surgical practice by comparing the technical feasibility, effectiveness, and safety of LTG and OTG in the treatment of early and advanced gastric cancer, through a systematic review and meta-analysis of published comparative studies.

MATERIALS AND METHODS

Literature search

A comprehensive literature search in Medline (PubMed), Embase, Academic Search Premier (EBSCO), Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library was carried out for relevant studies, between January 1994 and May 2013, comparing OTG and LTG in the treatment of gastric cancer. The following search terms were used: "gastric cancer; laparoscopic total gastrectomy; laparoscopic assisted total gastrectomy; minimally invasive surgery; open total gastrectomy" along with their synonyms or abbreviations. Reference lists of selected articles were also examined to identify relevant studies that were not identified in the database searches. Investigators and experts in the field of laparoscopic surgery were contacted to ensure that all relevant studies were identified. Only comparative clinical trials with full-text descriptions were included. Final inclusion of articles was determined by consensus; when this failed, a third author adjudicated.

Inclusion criteria

Studies included: (1) English language articles published in peer-reviewed journals; (2) human studies; (3) studies with at least one of the outcomes mentioned; (4) clear documentation of the operative techniques as "open" or "laparoscopic" or "laparoscopic-assisted"; and (5) where multiple studies came from the same institute and/or authors, either the higher quality study or the more recent publication was included in the analysis.

Exclusion criteria

Excluded studies: (1) abstracts, letters, editorials, expert opinions, case reports, reviews and studies lacking control groups; (2) studies for benign lesions and gastrointestinal stromal tumor (GIST); (3) studies comparing two laparoscopic surgical approaches or comparing laparoscopic and robot-assisted gastrectomy; (4) studies including only subgroup analyses comparing LTG with OTG; and (5) repeated reports between authors, centers, and the patient community.

Outcomes of interests

Operative outcomes included operation time and intraoperative blood loss. Oncological outcomes included positive resection margins, number of retrieved lymph nodes, and proximal and distal resection margins. Postoperative recovery outcomes included time to first flatus, time to first oral intake, analgesic use and hospital stay. Outcomes for postoperative complications included overall complication rate, anastomotic leakage, anastomotic stenosis, ileus, bleeding, abdominal abscess, wound-related prob-

lems and mortality.

Data extraction and quality assessment

Two independent observers using standardized forms extracted the data. The recorded data included study characteristics, quality assessment and perioperative outcomes. The quality of the studies was assessed using the modified Newcastle-Ottawa Scale, with changes made to reflect the needs of this study[20,21]. The maximum number of stars in the selection, comparability, and outcome categories were 3, 4, and 2, respectively. Studies achieving 6 or more stars were considered high quality[22].

Statistical analysis

Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). For continuous variables, treatment effects were expressed as weighted mean difference (WMD) with corresponding 95% confidence interval (CI). For categorical variables, treatment effects were expressed as odds ratio (OR) with corresponding 95%CI. Heterogeneity was evaluated using the χ^2 test, and a P value < 0.1 was considered significant; I^2 values were used for the evaluation of statistical heterogeneity[23]. A fixed-effects model was initially calculated for all outcomes[24], but if the test rejected the assumption of homogeneity of the studies, then a random-effects analysis was performed[25]. Sensitivity analyses were performed by removing individual studies from the data set and analyzing the effect on the overall results, to identify sources of significant heterogeneity. Subgroup analyses were also undertaken by including only high quality studies to present cumulative evidence. Funnel plots based on the operation time were constructed to evaluate potential publication bias[26].

RESULTS

Description of trials included in the meta-analysis

The search strategy generated 91 relevant clinical studies, among which 19 full text articles[9,10,14-18,27-38] were identified for further investigation. Of these, four studies[18,31,35,36] were excluded for various reasons: 1 study[36], based on an administrative database, was used to assess hospital practice performance with regard to the quantity of medical care items and diet provided during hospitalization; another study[35] only compared LTG with OTG in a subgroup analysis; and two studies were repeated reports[18,31]. Finally, 15 studies[9,10,14-17,27-30,32-34,37,38] were identified for inclusion, of which two were prospective non-randomized comparative studies[14,28], the rest being retrospective comparative studies. Figure 1 shows the study selection process in our meta-analysis.

Study and patient characteristics

Two thousand and twenty-two patients, 811 patients from the LTG group and 1211 patients from the OTG group, were included in the study. Eleven studies[9,10,14,16,17,28,29,32,34,37,38] included patients with both early and advanced gastric cancer, while three studies[15,30,33] only included patients with early gastric cancer; one study[27] only included patients with advanced gastric cancer. In seven studies[14,17,27,30,32,33,37], D2 lymph node dissection was exclusively performed, while D1+β was completed in three studies[9,15,28]. The remaining studies[10,16,29,34,38] reported D1+α/β and D2 dissections. All the studies were conducted in Asia and Europe, and were published between 2009 and 2013. The sample size ranged from 19 to 448 patients. From the nine studies[9,15,17,27,28,30,32,37,38] that reported data on conversion to an open procedure; LTG was converted to an open procedure in five patients in two studies[17,37]. The study characteristics (Table 1), quality assessment scoring (Table 2), perioperative outcomes of the included studies (Table 3) and the results of the meta-analysis (Table 4) have been summarized appropriately.

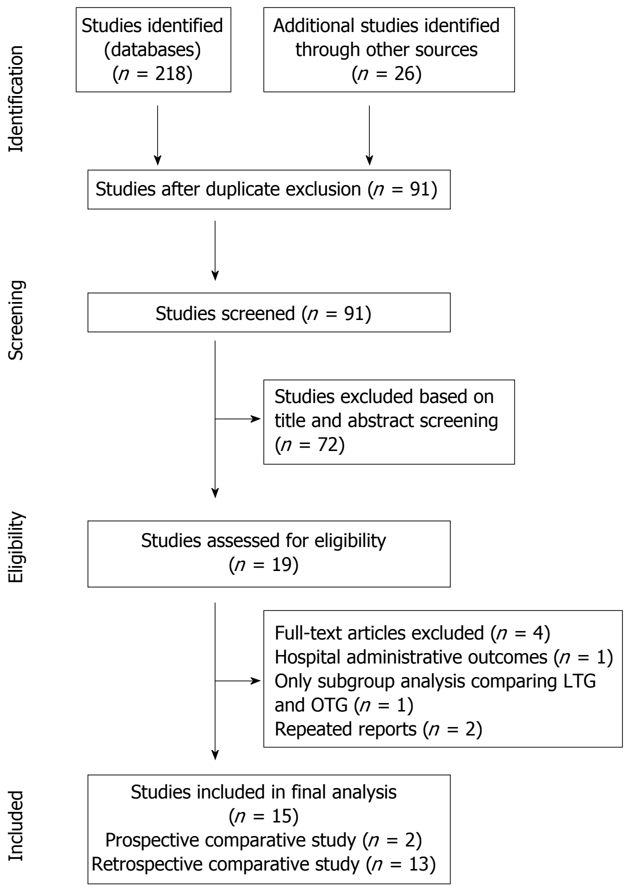


Figure 1 Flow diagram outlining the study selection process according to PRISMA guidelines. OTG: Open total gastrectomy; LTG: Laparoscopic total gastrectomy.

Operative outcomes

"Operation time" was reported in all studies. The analysis showed that the LTG group had a significantly longer operation time compared with the OTG group (WMD 48.25 min, 95%CI: 31.15-65.35, P < 0.00001), albeit with a significant heterogeneity (I^2 = 93%). Data from 12 studies[9,14-17,27-29,31,33,37,38] were pooled together to obtain the

Table 1 Study characteristics

Author, year	Country	Study design	Group	No. of patients	Age (yr)	Gender (M/F)	BMI (kg/m^2)	ASA (1:2:3)	Tumor size (cm)	Tumor stage[1]	Extent of LND	Population
Dulucq *et al*[28],	France	PCS	LTG	8	75 ± 8	3/5	NA	NA	5.5 ± 2	NA	D1 + β	EGC + AGC
2005			OTG	11	67 ± 14	5/6	NA	NA	6.1 ± 0.4	NA		
Usui *et al*[9],	Japan	RCS	LTG	20	66.0 ± 10.4	13/7	21.3 ± 3.1	NA	NA	8/10/2/0/0	D1 + β	EGC + AGC
2005			OTG	19	66.2 ± 10.2	14/5	22.1 ± 2.4	NA	NA	10/8/1/0/0		
Kim *et al*[34],	South	RCS	LTG	27	57.3 ± 14.2	16/11	22.6 ± 3.1	NA	NA	NA	D1 + α/β, D2	EGC + AGC
2008	Korea		OTG	33	61.6 ± 9.2	23/10	22.4 ± 2.1	NA	NA	NA		
Mochiki *et al*[15],	Japan	RCS	LTG	20	66 ± 2.4	16/4	NA	NA	3.6 ± 0.5	NA	D1 + β	EGC
2008			OTG	18	63 ± 2.2	16/2	NA	NA	5.7 ± 0.8	NA		
Topal *et al*[14],	Belgium	PCS	LTG	38	68 (37-85)	23/15	24 (17-30)	NA	47 (7-180)	0/17/7/10/4	D2	EGC + AGC
2008			OTG	22	69 (38-86)	17/5	24 (17-30)	NA	30 (10-180)	0/7/7/6/2		
Kawamura *et al*[30],	Japan	RCS	LTG	46	64 ± 10.4	10/36	22.8 ± 3.0	15/27/4	NA	NA	D2	EGC
2009			OTG	35	65.2 ± 10.7	10/25	22.9 ± 2.4	14/15/6	NA	NA		
Sakuramoto *et al*[16],	Japan	RCS	LTG	30	63.7 ± 9.2	12/18	21.9 ± 2.7	9/20/1	4.0 ± 2.9	0/25/2/3/0	D1 + β, D2	EGC + AGC
2009			OTG	44	67.2 ± 9.9	10/34	22.5 ± 3.6	8/28/8	6.1 ± 3.7	0/15/17/12/0		
Du *et al*[27],	China	RCS	LTG	82	60.4 ± 18.5	54/28	22.3 ± 2.6	NA	5.4 ± 1.4	0/3/36/43/0	D2	AGC
2010			OTG	94	57.8 ± 17.2	61/33	22.5 ± 2.4	NA	5.9 ± 1.6	0/6/31/57/0		
Kim *et al*[33],	South	RCS	LTG	63	55.9 ± 12.2	43/20	22.7 ± 2.5	45/15/3	3.8 ± 2.1	NA	D2	EGC
2011	Korea		OTG	127	57.3 ± 11.1	81/46	23.0 ± 2.9	86/39/2	3.9 ± 2.7	NA		
Eom *et al*[10],	South	RCS	LTG	100	54.9 ± 13.5	57/43	22.7 ± 2.8	NA	4.3 ± 2.9	NA	D1 + β, D2	EGC + AGC
2012	Korea		OTG	348	58.7 ± 11.5	254/94	23.8 ± 2.9	NA	4.4 ± 3.0	NA		
Guan *et al*[17],	China	RCS	LTG	41	60.7 ± 9.1	33/8	NA	NA	NA	0/18/20/3/0	D2	EGC + AGC
2012			OTG	56	57.8 ± 9.9	40/16	NA	NA	NA	0/25/25/6/0		
Siani *et al*[38],	Italy	RCS	LTG	25	65 ± 8.5	15/10	NA	NA	NA	0/6/5/14/0	D1 + α/β, D2	EGC + AGC
2012			OTG	25	66 ± 7.8	18/7	NA	NA	NA	0/4/5/16/0		
Kim *et al*[32],	South	RCS	LTG	139	58 (30-84)	86/53	23.6 (13.6-32.4)	85/46/8	3.2 (0.2, 15)	NA	D2	EGC + AGC
2013	Korea		OTG	207	56 (31-84)	134/73	24.1 (16.7-35.2)	137/52/18	4.0 (0.3, 22)	NA		
Jeong *et al*[29],	South	RCS	LTG	122	63.2 ± 11.2	89/33	23.1 ± 3.4	33/80/9	NA	NA	D1 + β, D2	EGC + AGC
2013	Korea		OTG	122	62.6 ± 11.7	93/29	23.5 ± 3.2	43/67/12	NA	NA		
Lee *et al*[37],	South	RCS	LTG	50	50.6 ± 22.1	32/18	23.2 ± 3.7	34/11/5	NA	0/24/13/9/4	D2	EGC + AGC
2013	Korea		OTG	50	51 ± 22.6	32/18	23 ± 3.4	31/16/3	NA	0/24/13/9/4		

Continuous variables are presented as means ± SD or median and range. [1]Pathological tumor stage (0/Ⅰ/Ⅱ/Ⅲ/Ⅳ). PCS: Prospective comparative study; RCS: Retrospective comparative study; LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy; BMI: Body mass index; NA: Not available; ASA: American Society of Anesthesiologists; LND: Lymph node dissection; EGC: Early gastric cancer; AGC: Advanced gastric cancer; M/F: Male/female.

mean intraoperative blood loss in the two groups. LTG was associated with a significantly lower intraoperative blood loss compared with OTG (WMD -201.19 mL, 95%CI: -296.50--105.87 mL, P < 0.0001), with a significant heterogeneity (I^2 = 98%). Forest plots for operative outcomes are shown in Figure 2.

Postoperative recovery

Twelve studies[9,16,17,27-30,32-34,37,38] reported the time to first flatus and eight studies[9,16,17,27,29,32,33,37] reported data on oral intake post-surgery. Our analyses showed that patients undergoing LTG had a quicker recovery of intestinal motility compared with the OTG group. The time to first flatus (WMD -0.82, 95%CI: -1.18--0.45, P < 0.0001) and the time to first oral intake (WMD -1.30, 95%CI: -1.84--0.75, P < 0.00001) were significantly shorter in the LTG group compared with the OTG group. Analysis of the 13 studies[9,10,15-17,28-30,32-34,37,38] that reported the duration of hospital stay indicated that LTG was associated with a significantly shorter postoperative hospital stay compared with OTG (WMD -3.55, 95%CI: -5.13--1.96, P < 0.0001). However, there was no statistically significant difference between the two groups in the use of analgesics post-surgery (WMD -0.09, 95%CI: -2.39-2.20, P = 0.94). Forest plots for postoperative recovery outcomes are shown in Figure 3.

Postoperative complications

A pooled analysis of 14 studies[10,14-17,27-30,32-34,37,38] indicated that the overall complication rate was significantly lower in the LTG group compared with the OTG group (OR = 0.73, 95%CI: 0.57-0.92, P = 0.009). Also, the analysis of 13 studies[10,15-17,27-30,32-34,37,38] suggested that patients in the LTG group had significantly fewer wound-related complications compared with the OTG group (OR = 0.39, 95%CI: 0.21-0.72, P = 0.002). However, there were no significant differences in the rate of anastomotic leak (OR = 1.6, 95%CI: 0.88-2.91, P = 0.12), anastomotic stenosis (OR = 1.22, 95%CI: 0.68-2.21, P = 0.50), ileus (OR 1.26, 95%CI: 0.69,2.30; P = 0.46), bleeding (OR = 1.42, 95%CI: 0.70-2.87; P = 0.33), abdominal abscess (OR = 0.53, 95%CI: 0.28-1.03, P = 0.06) or mortality (OR = 0.74, 95%CI: 0.24-2.31, P = 0.61) between the two groups. Forest plots for postoperative outcomes are shown in Figure 4.

Oncological outcomes

All included studies reported data on the number of lymph nodes retrieved; there was no significant difference between the two groups (WMD -2.49, 95%CI: -5.18-0.21, P = 0.07), albeit with a significant heterogeneity in the result (I^2 = 74%). Five studies[14,17,27,28,32] reported

Table 2 Quality assessment scoring of included studies, according to NOS criterion

Author, year	Selection			Comparability[1]		Outcome assessment		Star Score
	1	2	3	4	5	6	7	
Dulucq *et al*[28], 2005	*	*	*	*		*	*	******
Usui *et al*[9], 2005	*	*	*			*	*	*****
Kim *et al*[34], 2008	*	*	*			*		****
Mochiki *et al*[15], 2008	*	*	*	*		*	*	******
Topal *et al*[14], 2008	*	*	*	**	**	*		********
Kawamura *et al*[30], 2009	*	*	*	**	*	*	*	********
Sakuramoto *et al*[16], 2009	*	*	*	**		*	*	*******
Du *et al*[27], 2010	*	*	*	**		*	*	*******
Kim *et al*[33], 2011	*	*	*	**	*	*		*******
Eom *et al*[10], 2012	*	*	*			*	*	*****
Guan *et al*[17], 2012	*	*	*		*	*		*****
Siani *et al*[38], 2012	*	*	*	*	*	*	*	*******
Kim *et al*[32], 2013	*	*	*	**		*		******
Jeong *et al*[29], 2013	*	*	*	**	*	*		*******
Lee *et al*[37], 2013	*	*	*	**	*	*	*	********

Based on Newcastle-Ottowa Scale with maximum of *** for selection, ****for comparability, and ** for outcome assessment. [1]Comparability variables are (1) age, (2) sex, (3) body mass index, (4) American Society of Anesthesiologists, (5) comorbidity, (6) tumor size and (7) tumor stage. Group comparable for (1)-(3) or (4)-(7) (if yes, two stars, one star if one of these three characteristics was not reported, even if there were no other differences between the two groups and other characteristics had been controlled; no points were assigned if the two groups differed).

Table 3 Perioperative outcomes

Author, year	Group	Operation time (min)	Intraoperative blood loss (mL)	No. of resected lymph nodes (*n*)	Time to first flatus (d)	Time to first oral intake (d)	Hospital stay (d)	Analgesics use (times)	Postoperative complications (%)	In-hospital Mortality (%)
Dulucq *et al*[28],	LTG	183 ± 48	81 ± 107	24 ± 12	3.6 ± 1.2	NA	16.9 ± 3	NA	0	0
2005	OTG	165 ± 60	125 ± 95	20 ± 8	4.7 ± 1.2	NA	24 ± 9	NA	18	9
Usui *et al*[9],	LTG	280.1 ± 45.2	227.5 ± 148.1	28.0 ± 15.1	2.9 ± 0.9	5.7 ± 2.1	15.5 ± 3.9	2.1 ± 1.3	NA	NA
2005	OTG	266.4 ± 48.2	393.1 ± 173.6	28.9 ± 14.3	4.2 ± 1.4	8.8 ± 1.3	23.2 ± 4.6	3.4 ± 4.4	NA	NA
Kim *et al*[34],	LTG	527.5 ± 95.7	NA	27.2 ± 15.7	3.6 ± 0.9	NA	16.2 ± 7.1	NA	7.4	0
2008	OTG	320.9 ± 75.8	NA	37.2 ± 15.7	4.1 ± 1.3	NA	16.0 ± 9.3	NA	24.2	0
Mochiki *et al*[15],	LTG	254 ± 10	299 ± 50	26 ± 3	NA	NA	19 ± 3	NA	25	0
2008	OTG	248 ± 12	758 ± 78	35 ± 4	NA	NA	29 ± 3	NA	16.7	0
Topal *et al*[14],	LTG	187 ± 60	10.0 ± 98.8	NA	NA	NA	NA	NA	39.5	2.6
2008	OTG	152.5 ± 25	450.0 ± 337.5	NA	NA	NA	NA	NA	40.9	4.5
Kawamura *et al*[30],	LTG	291.9 ± 59.4	54.9 ± 45.3	48.5 ± 16.3	4.1 ± 1.0	NA	15.5 ± 3.3	6.9 ± 5.6	8.7	0
2009	OTG	272.1 ± 76.8	304.3 ± 237.3	47.1 ± 21.5	4.3 ± 1.3	NA	18.8 ± 6.3	4.0 ± 3.2	22.9	0
Sakuramoto *et al*[16],	LTG	313 ± 81	134 ± 98	43.2 ± 17.2	2.4 ± 1.1	4.9 ± 1.1	13.5 ± 2.7	6.8 ± 6.4	16.7	0
2009	OTG	218 ± 53	407 ± 270	51.2 ± 22.1	3.3 ± 1.0	6.0 ± 2.1	18.2 ± 9.6	11.8 ± 11.0	27.3	0
Du *et al*[27],	LTG	275 ± 78	156 ± 112	34.2 ± 13.5	3.5 ± 0.8	3.5 ± 0.8	NA	NA	9.8	0
2010	OTG	212 ± 51	339 ± 162	36.4 ± 19.1	5.3 ± 1.3	5.3 ± 1.3	NA	NA	24.5	2.1
Kim *et al*[33],	LTG	150.8 ± 31.2	179.7 ± 123.8	38.7 ± 15.7	3.3 ± 0.7	4.3 ± 1.7	8.1 ± 3.8	5.3 ± 4.9	12.7	0
2011	OTG	131.2 ± 21.6	272.7 ± 209.6	35.6 ± 13.1	3.8 ± 0.8	5.6 ± 4.4	9.6 ± 5.3	3.6 ± 3.9	18.9	0
Eom *et al*[10],	LTG	283.7 ± 84.1	NA	48.3 ± 16.4	NA	NA	12.6 ± 15.5	NA	27	1
2012	OTG	198.5 ± 59.7	NA	49.8 ± 18.4	NA	NA	14.3 ± 16.7	NA	23.6	0.9
Guan *et al*[17],	LTG	235.7 ± 38.5	104.2 ± 42.9	23.1 ± 8.0	3 ± 0.7	2.2 ± 0.9	9.7 ± 2.2	NA	4.9	0
2012	OTG	211.5 ± 33.2	355.6 ± 51.3	24.2 ± 7.5	3.3 ± 0.4	3.1 ± 0.5	13.6 ± 3.6	NA	5.4	0
Siani *et al*[38],	LTG	211 ± 23	250 ± 150	35 ± 18	2.1 ± 0.9	NA	10.5 ± 1.5	NA	16	0
2012	OTG	185 ± 19	495 ± 190	40 ± 16	4.1 ± 1.5	NA	14.5 ± 3.1	NA	4	0
Kim *et al*[32],	LTG	144 ± 104.3	NA	37 ± 24	3 ± 2	3 ± 12.3	7 ± 19.3	3 ± 24.5	10	0
2013	OTG	137 ± 105	NA	34 ± 18.8	4 ± 2.3	5 ± 10	8 ± 9	4 ± 9.3	21.7	0
Jeong *et al*[29],	LTG	289 ± 89	249 ± 204	42 ± 15	2.9 ± 0.8	3.9 ± 4.4	11.8 ± 11.8	NA	23.8	1.6
2013	OTG	203 ± 78	209 ± 157	46 ± 17	3.0 ± 0.8	3.6 ± 3.3	10.8 ± 7.0	NA	17.2	0.9
Lee *et al*[37],	LTG	258 ± 54	167.3 ± 135.2	48.4 ± 18.4	4 ± 1.2	5 ± 1.7	9.3 ± 4.2	NA	24	0
2013	OTG	198 ± 57	178.4 ± 107	54.3 ± 20.5	4.5 ± 1.5	6.1 ± 2.5	11.7 ± 7.3	NA	32	0

LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy; NA: Not available.

data on positive resection margins; in only one study[14], resection margins were found to be positive in one patient each from the LTG and OTG groups and with no significant difference between the two groups (OR = 0.57, 95%CI: 0.03-9.55, P = 0.69). There were also no significant differences in the lengths of the proximal resection margin (WMD -0.26, 95%CI: -0.54-0.01, P = 0.06) and distal resection margin (WMD 0.32, 95%CI: -0.05-0.68, P = 0.09) between the two groups when data from four studies[10,27,32,33] were pooled. Seven studies re-

Table 4 Results of meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy

Outcome of interest	No. of studies	No. of patients	OR/WMD	95%CI	*P* value	Heterogeneity *P* value	I^2
Operative outcomes							
Operation time (min)	15	2022	48.25	31.15-65.35	< 0.00001	< 0.00001	93%
Intraoperative blood loss (mL)	12	1168	-201.19	-296.50--105.87	< 0.0001	< 0.00001	98%
Postoperative recovery							
Time to first flatus (d)	12	1412	-0.82	-1.18--0.45	< 0.0001	< 0.00001	90%
Time to first oral intake (d)	8	1266	-1.3	-1.84--0.75	< 0.00001	< 0.00001	82%
Hospital stay (d)	13	1786	-3.55	-5.13--1.96	< 0.0001	< 0.00001	86%
Analgesics use (times)	5	730	-0.09	-2.39-2.20	0.94	0.0008	79%
Postoperative complications							
Overall complication	14	1983	0.73	0.57-0.92	0.009	0.08	37%
Anastomotic leakage	14	1983	1.6	0.88-2.91	0.12	0.68	0%
Anastomotic stenosis	13	1923	1.22	0.68-2.21	0.50	0.95	0%
Ileus	13	1923	1.26	0.69-2.30	0.46	0.85	0%
Bleeding	13	1923	1.42	0.70-2.87	0.33	0.26	23%
Abdominal abscess	13	1923	0.53	0.28-1.03	0.06	0.37	8%
Wound problems	13	1923	0.39	0.21-0.72	0.002	0.75	0%
Oncological outcomes							
Positive resection margins	5	698	0.57	0.03-9.55	0.69	-	-
No. of resected lymph nodes	14	1962	-2.49	-5.18-0.21	0.07	< 0.00001	74%
Proximal resection margin (cm)	4	1160	-0.26	-0.54-0.01	0.06	0.65	0%
Distal resection margin (cm)	4	1160	0.32	-0.05-0.68	0.09	0.22	32%

ported data on long-term survival following the two procedures[10,15,16,27,28,37,38]. Lee *et al*[37] reported no significant difference in the disease-specific survival rate between the LTG and OTG groups at a median follow-up of 50 months; there were also no significant differences reported in the disease-free survival rate (100% *vs* 90.9%, $P = 0.5$) and the cumulative survival rate (91.5% *vs* 95.2%, $P = 0.618$) in patients with stage I cancer (TNM) between the LTG and OTG groups. Eom *et al*[10] reported no significant difference in the disease-free survival rates between the LTG and OTG groups, after adjustment for five variables (age, tumor size, Lauren classification, depth of invasion and lymph node metastasis). Mochiki *et al*[15] reported no significant difference in the cumulate 5-year or disease-specific survival rates between the LTG and OTG groups, while Siani *et al*[38] reported 5-year overall and disease free survival rates of 55.7% and 54.2% in the LTG group and 52.9% and 52.1% in the OTG group respectively, with no statistically significant differences. However, as the duration of follow-up varied between studies, it was difficult to compare the survival rates. Forest plots for oncological outcomes are shown in Figure 5.

Sensitivity and subgroup analysis

Sensitivity analyses were performed by removing individual studies from the data and analyzing the effect on the overall results to identify sources of significant heterogeneity. These exclusions did not alter the results obtained from the cumulative analyses. Subgroup analyses were undertaken for all outcome measures by including only high quality studies. Analysis of the high-quality studies showed that there were no significant differences for any of the outcomes. These are shown in Figure 6.

Publication bias

The funnel plot based on the operation time is shown in Figure 7. There was no broad evidence of publication bias, as none of the studies lay outside the 95%CI limits.

DISCUSSION

Laparoscopic surgery is being used increasingly to treat gastric cancer, and has been shown to have many advantages over open surgery. However, LTG is less widely practiced compared with laparoscopic distal gastrectomy because of the technical challenges it poses and the absence of compelling evidence to substantiate its use[9]. Technical advances, better instrumentation and increasing surgical experience in the procedure are aiding its increasing application to treat of early and advanced gastric cancer. The aim of the current study was to inform future surgical practice by comparing the technical feasibility, effectiveness, and safety of LTG with OTG in the treatment of early and advanced gastric cancer, using a systematic review and meta-analysis of published comparative studies.

Our analyses indicated that the operation time was significantly longer in the LTG group than in the OTG group. This may be because LTG is more technically demanding than OTG and may result from the learning curve associated with the procedure[9,10,35]. While adequate training in laparoscopic techniques is necessary, it was concluded that an experienced laparoscopic surgeon would not require any more time to perform LTG compared with OTG[15]. In one study, the operation time for LTG in the later period was significantly shorter than in the early period; this related to the experience gained by the surgeon over the period of the study[34]. Further development in surgical techniques, especially for anastomosis and new instruments, may further decrease the operation time for LTG[10]. In our study, LTG was associated with a significantly lower intraoperative blood

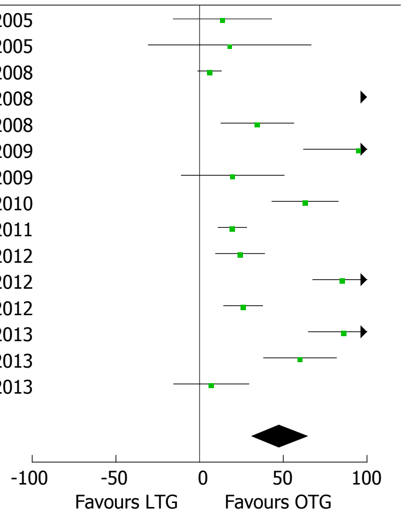
A

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Usui 2005	280.1	45.2	20	266.4	48.2	19	6.3%	13.70 [-15.66, 43.06]	2005
Dulucq 2005	183	48	8	165	60	11	4.8%	18.00 [-30.62, 66.62]	2005
Mochiki 2008	254	10	20	248	12	18	7.6%	6.00 [-1.07, 13.07]	2008
Kim 2008	527.5	95.7	27	320.9	75.8	33	5.1%	206.60 [162.19, 251.01]	2008
Topal 2008	187	60	38	152.5	25	22	6.9%	34.50 [12.75, 56.25]	2008
Sakuramoto 2009	313	81	30	218	53	44	6.0%	95.00 [62.06, 127.94]	2009
Kawamura 2009	291.9	59.4	46	272.1	76.8	35	6.2%	19.80 [-10.89, 50.49]	2009
Du 2010	275	78	82	212	51	94	7.0%	63.00 [43.22, 82.78]	2010
Kim 2011	150.8	31.2	63	131.2	21.6	127	7.6%	19.60 [11.03, 28.17]	2011
Guan 2012	235.7	38.5	41	211.5	33.2	56	7.3%	24.20 [9.55, 38.85]	2012
Eom 2012	283.7	84.1	100	198.5	59.7	348	7.1%	85.20 [67.56, 102.84]	2012
Siani 2012	211	23	25	185	19	25	7.5%	26.00 [14.31, 37.69]	2012
Jeong 2013	289	89	122	203	78	122	6.9%	86.00 [65.00, 107.00]	2013
Lee 2013	258	54	50	198	57	50	6.9%	60.00 [38.24, 81.76]	2013
Kim 2013	144	104.3	139	137	105	207	6.8%	7.00 [-15.48, 29.48]	2013
Total (95%CI)			811			1211	100.0%	48.25 [31.15, 65.35]	

Heterogeneity: Tau² = 984.69; χ^2 = 214.38, *df* = 14 (*P* < 0.00001); I^2 = 93%

Test for overall effect: *Z* = 5.53 (*P* < 0.00001)

Mean difference IV, random, 95%CI



B

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Usui 2005	227.5	148.1	20	393.1	173.6	19	8.0%	-165.60 [-267.12, -64.08]	2005
Dulucq 2005	81	107	8	125	95	11	8.1%	-44.00 [-137.00, 49.00]	2005
Mochiki 2008	229	50	20	758	78	18	8.7%	-529.00 [-571.17, -486.83]	2008
Topal 2008	10	98.8	38	450	337.5	22	7.3%	-440.00 [-584.49, -295.51]	2008
Kawamura 2009	54.9	45.3	46	304.3	237.3	35	8.3%	-249.40 [-329.10, -169.70]	2009
Sakuramoto 2009	134	98	30	407	270	44	8.2%	-273.00 [-360.15, -185.85]	2009
Du 2010	156	112	82	339	162	94	8.7%	-183.00 [-223.74, -142.26]	2010
Kim 2011	179.7	123.8	63	272.7	209.6	127	8.6%	-93.00 [-140.58, -45.42]	2011
Guan 2012	104.2	42.9	41	355.6	51.3	56	8.8%	-251.40 [-270.19, -232.61]	2012
Siani 2012	250	150	25	495	190	25	8.1%	-245.00 [-339.89, -150.11]	2012
Lee 2013	167.3	135.2	50	178.4	107	50	8.6%	-11.10 [-58.89, 36.69]	2013
Jeong 2013	249	204	122	209	157	122	8.6%	40.00 [-5.68, 85.68]	2013
Total (95%CI)			545			623	100.0%	-201.19 [-296.50, -105.87]	

Heterogeneity: Tau² = 26859.21; χ^2 = 470.65, *df* = 11 (*P* < 0.00001); I^2 = 98%

Test for overall effect: *Z* = 4.14 (*P* < 0.0001)

Mean difference IV, random, 95%CI

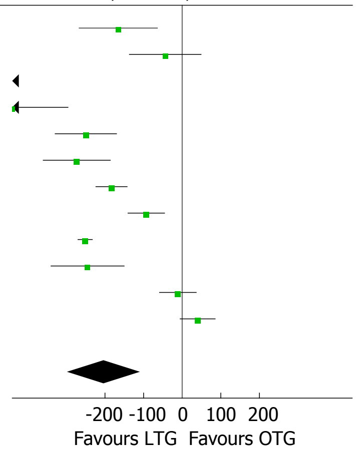


Figure 2 Forest plots illustrating results of operative outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) with 95%CI was calculated using the random effects model. A: Operation time; B: Intraoperative blood loss. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

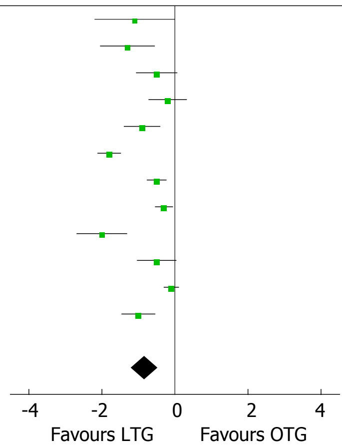
A

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	3.6	1.2	8	4.7	1.2	11	5.3%	-1.10 [-2.19, -0.01]	2005
Usui 2005	2.9	0.9	20	4.2	1.4	19	7.1%	-1.30 [-2.04, -0.56]	2005
Kim 2008	3.6	0.9	27	4.1	1.3	33	8.1%	-0.50 [-1.06, 0.06]	2008
Kawamura 2009	4.1	1	46	4.3	1.3	35	8.4%	-0.20 [-0.72, 0.32]	2009
Sakuramoto 2009	2.4	1.1	30	3.3	1	44	8.5%	-0.90 [-1.39, -0.41]	2009
Du 2010	3.5	0.8	82	5.3	1.3	94	9.3%	-1.80 [-2.11, -1.49]	2010
Kim 2011	3.3	0.7	63	3.8	0.8	63	9.5%	-0.50 [-0.76, -0.24]	2011
Guan 2012	3	0.7	41	3.3	0.4	56	9.6%	-0.30 [-0.54, -0.06]	2012
Siani 2012	2.1	0.9	25	4.1	1.5	25	7.4%	-2.00 [-2.69, -1.31]	2012
Lee 2013	4	1.2	50	4.5	1.5	50	8.3%	-0.50 [-1.03, 0.03]	2013
Jeong 2013	2.9	0.8	122	3	0.8	122	9.7%	-0.10 [-0.30, 0.10]	2013
Kim 2013	3	2	139	4	2.3	207	8.7%	-1.00 [-1.46, -0.54]	2013
Total (95%CI)			653			759	100.0%	-0.82 [-1.18, -0.45]	

Heterogeneity: Tau² = 0.35; χ^2 = 113.38, *df* = 11 (*P* < 0.00001); I^2 = 90%

Test for overall effect: *Z* = 4.37 (*P* < 0.0001)

Mean difference IV, random, 95%CI

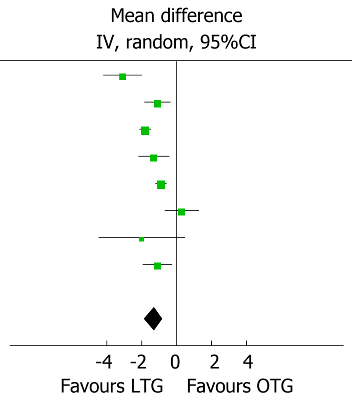


B

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Usui 2005	5.7	2.1	20	8.8	1.3	19	10.6%	-3.10 [-4.19, -2.01]	2005
Sakuramoto 2009	4.9	1.1	30	6	2.1	44	13.8%	-1.10 [-1.83, -0.37]	2009
Du 2010	3.5	0.8	82	5.3	1.3	94	17.4%	-1.80 [-2.11, -1.49]	2010
Kim 2011	4.3	1.7	63	5.6	4.4	127	12.5%	-1.30 [-2.17, -0.43]	2011
Guan 2012	2.2	0.9	41	3.1	0.5	56	17.4%	-0.90 [-1.21, -0.59]	2012
Jeong 2013	3.9	4.4	122	3.6	3.3	122	11.6%	0.30 [-0.68, 1.28]	2013
Kim 2013	3	12.3	139	5	10	207	3.9%	-2.00 [-4.46, 0.46]	2013
Lee 2013	5	1.7	50	6.1	2.5	50	12.8%	-1.10 [-1.94, -0.26]	2013
Total (95%CI)			547			719	100.0%	-1.30 [-1.84, -0.75]	

Heterogeneity: Tau² = 0.42; χ^2 = 37.91, *df* = 7 (P < 0.00001); I^2 = 82%

Test for overall effect: Z = 4.67 (P < 0.00001)

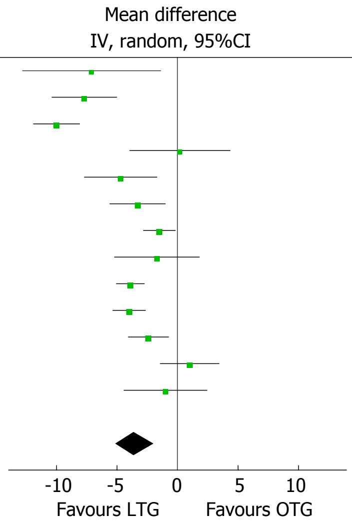


C

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	16.9	3	8	24	9	11	4.3%	-7.10 [-12.81, -1.39]	2005
Usui 2005	15.5	3.9	20	23.2	4.6	19	7.7%	-7.70 [-10.38, -5.02]	2005
Mochiki 2008	19	3	20	29	3	18	8.6%	-10.00 [-11.91, -8.09]	2008
Kim 2008	16.2	7.1	27	16	9.3	33	5.9%	0.20 [-3.95, 4.35]	2008
Sakuramoto 2009	13.5	2.7	30	18.2	9.6	44	7.3%	-4.70 [-7.70, -1.70]	2009
Kawamura 2009	15.5	3.3	46	18.8	6.3	35	8.2%	-3.30 [-5.59, -1.01]	2009
Kim 2011	8.1	3.8	63	9.6	5.3	127	9.2%	-1.50 [-2.82, -0.18]	2011
Eom 2012	12.6	15.5	100	14.3	16.7	348	6.6%	-1.70 [-5.21, 1.81]	2012
Guan 2012	9.7	2.2	41	13.6	3.6	56	9.4%	-3.90 [-5.06, -2.74]	2012
Siani 2012	10.5	1.5	25	14.5	3.1	25	9.2%	-4.00 [-5.35, -2.65]	2012
Lee 2013	9.3	4.2	50	11.7	4.3	50	8.9%	-2.40 [-4.07, -0.73]	2013
Jeong 2013	11.8	11.8	122	10.8	7	122	8.0%	1.00 [-1.43, 3.43]	2013
Kim 2013	7	19.3	139	8	9	207	6.7%	-1.00 [-4.43, 2.43]	2013
Total (95%CI)			691			1095	100.0%	-3.55 [-5.13, -1.96]	

Heterogeneity: Tau² = 6.66; χ^2 = 86.74, *df* = 12 (P < 0.00001); I^2 = 86%

Test for overall effect: Z = 4.38 (P < 0.0001)



D

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Usui 2005	2.1	1.3	20	3.4	4.4	19	22.6%	-1.30 [-3.36, 0.76]	2005
Sakuramoto 2009	6.8	6.4	30	11.8	11	44	15.1%	-5.00 [-8.98, -1.02]	2009
Kawamura 2009	6.9	5.6	46	4	3.2	35	23.1%	2.90 [0.97, 4.83]	2009
Kim 2011	5.3	4.9	63	3.6	3.9	127	25.1%	1.70 [0.31, 3.09]	2011
Kim 2013	3	24.5	139	4	9.3	207	14.1%	-1.00 [-5.27, 3.27]	2013
Total (95%CI)			298			432	100.0%	-0.09 [-2.39, 2.20]	

Heterogeneity: Tau² = 4.96; χ^2 = 18.98, *df* = 4 (P = 0.0008); I^2 = 79%

Test for overall effect: Z = 0.08 (P = 0.94)

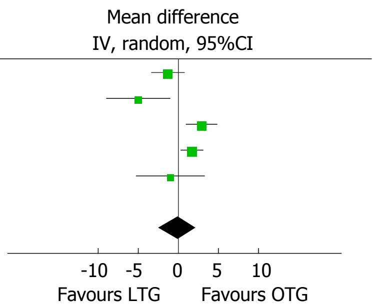


Figure 3 Forest plots illustrating results of postoperative recovery in the form of a meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) with 95%CI was calculated using the random-effects model. A: Time to first flatus; B Time to first oral intake; C: Hospital stay; D: Analgesic use. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

loss, which depends considerably on a surgeon's skill and experience[34].

The times to first flatus and to first oral intake were significantly shorter in the LTG group compared with the OTG group, which suggests that intestinal motility recovered more quickly in the LTG group. Also, the period of hospital stay was significantly shorter in the LTG group. LTG is a less invasive procedure and is associated with less surgical trauma. This results in a reduced inflammatory response and better glucose tolerance, which may aid a quicker recovery[19,30]. Pain following LTG subsides earlier when compared to OTG[18]. However, our study showed no significant difference in the postoperative use of analgesics between the two groups. A quicker recovery and shorter hospital stay have important cost and quality of life implications for the wider use of LTG in the treatment of gastric cancer.

Total gastrectomy has often been described as high-risk[39,40] and LTG is technically demanding[9,10]. Common postoperative complications associated with LTG include anastomotic leak, anastomotic stenosis and luminal bleeding[37]. The anastomotic complications could be caused by excessive traction applied on the esophagus and jejunal limb mobilization[10] or may reflect the learning curve associated with LTG[37]. In our study, the overall complication rate was significantly lower in the LTG group compared with the OTG group. Also, there were significantly fewer wound-related complications in the LTG

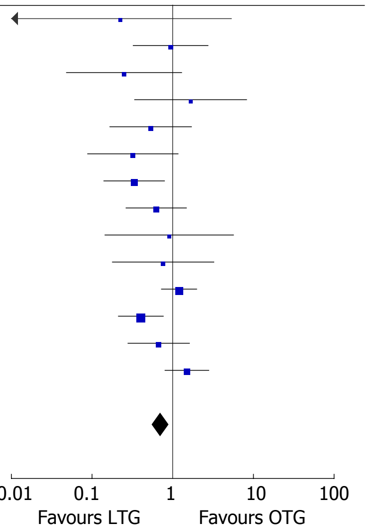
A

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	2	11	1.3%	0.22 [0.01, 5.34]	2005
Topal 2008	15	38	9	22	4.3%	0.94 [0.32, 2.75]	2008
Mochiki 2008	5	20	3	18	1.5%	1.67 [0.34, 8.26]	2008
Kim 2008	2	27	8	33	4.1%	0.25 [0.05, 1.30]	2008
Sakuramoto 2009	5	30	12	44	5.0%	0.53 [0.17, 1.71]	2009
Kawamura 2009	4	46	8	35	5.1%	0.32 [0.09, 1.17]	2009
Du 2010	8	82	23	94	12.0%	0.33 [0.14, 0.79]	2010
Kim 2011	8	63	24	127	8.6%	0.62 [0.26, 1.48]	2011
Guan 2012	2	41	3	56	1.5%	0.91 [0.14, 5.68]	2012
Siani 2012	4	25	5	25	2.6%	0.76 [0.18, 3.25]	2012
Eom 2012	27	100	82	348	16.5%	1.20 [0.72, 1.99]	2012
Kim 2013	14	139	45	207	20.1%	0.40 [0.21, 0.77]	2013
Lee 2013	12	50	16	50	7.5%	0.67 [0.28, 1.62]	2013
Jeong 2013	29	122	21	122	9.9%	1.50 [0.80, 2.81]	2013
Total (95%CI)		791		1192	100.0%	0.73 [0.57, 0.92]	

Heterogeneity: $\chi^2 = 20.60$, $df = 13$ ($P = 0.08$); $I^2 = 37\%$

Test for overall effect: $Z = 2.62$ ($P = 0.009$)

Odds Ratio M-H, fixed, 95%CI



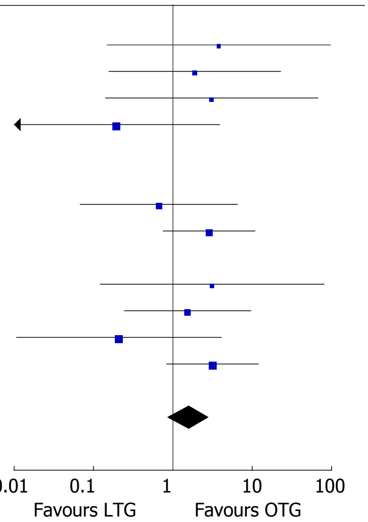
B

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Kim 2008	1	27	0	33	2.5%	3.79 [0.15, 96.92]	2008
Mochiki 2008	2	20	1	18	5.6%	1.89 [0.16, 22.79]	2008
Topal 2008	2	38	0	22	3.5%	3.08 [0.14, 67.16]	2008
Sakuramoto 2009	0	30	3	44	16.7%	0.19 [0.01, 3.90]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	1	63	3	127	11.6%	0.67 [0.07, 6.54]	2011
Eom 2012	4	100	5	348	12.7%	2.86 [0.75, 10.85]	2012
Guan 2012	0	41	0	56		Not estimable	2012
Siani 2012	1	25	0	25	2.8%	3.12 [0.12, 80.39]	2012
Lee 2013	3	50	2	50	11.2%	1.53 [0.24, 9.59]	2013
Kim 2013	0	139	3	207	16.7%	0.21 [0.01, 4.09]	2013
Jeong 2013	9	122	3	122	16.5%	3.16 [0.83, 11.97]	2013
Total (95%CI)		791		1192	100.0%	1.60 [0.88, 2.91]	

Heterogeneity: $\chi^2 = 6.62$, $df = 9$ ($P = 0.68$); $I^2 = 0\%$

Test for overall effect: $Z = 1.54$ ($P = 0.12$)

Odds ratio M-H, fixed, 95%CI



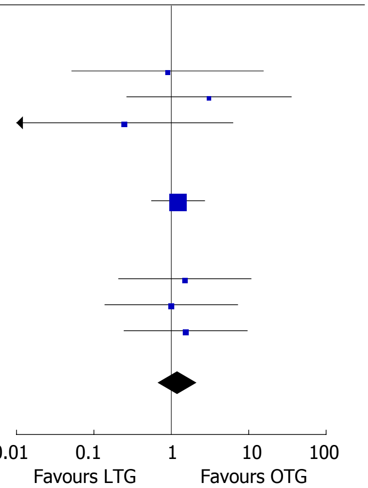
C

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Kim 2008	0	27	0	33		Not estimable	2008
Mochiki 2008	1	20	1	18	5.1%	0.89 [0.05, 15.44]	2008
Sakuramoto 2009	2	30	1	44	3.9%	3.07 [0.27, 35.49]	2009
Kawamura 2009	0	46	1	35	8.6%	0.25 [0.01, 6.26]	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	0	63	0	127		Not estimable	2011
Eom 2012	9	100	26	348	54.4%	1.22 [0.55, 2.71]	2012
Siani 2012	0	25	0	25		Not estimable	2012
Guan 2012	0	41	0	56		Not estimable	2012
Kim 2013	2	139	2	207	8.2%	1.50 [0.21, 10.75]	2013
Jeong 2013	2	122	2	122	10.1%	1.00 [0.14, 7.22]	2013
Lee 2013	3	50	2	50	9.7%	1.53 [0.24, 9.59]	2013
Total (95%CI)		753		1170	100.0%	1.22 [0.68, 2.21]	

Heterogeneity: $\chi^2 = 1.67$, $df = 6$ ($P = 0.95$); $I^2 = 0\%$

Test for overall effect: $Z = 0.67$ ($P = 0.50$)

Odds ratio M-H, fixed, 95%CI

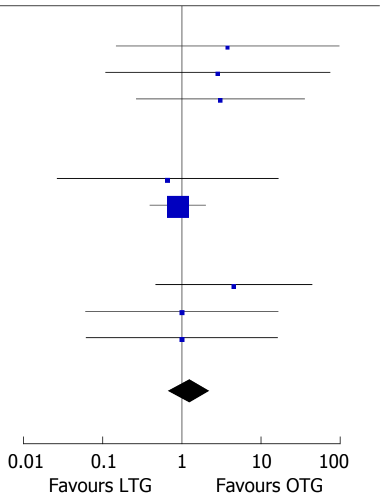


D

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Kim 2008	1	27	0	33	2.4%	3.79 [0.15, 96.92]	2008
Mochiki 2008	1	20	0	18	2.7%	2.85 [0.11, 74.38]	2008
Sakuramoto 2009	2	30	1	44	4.2%	3.07 [0.27, 35.49]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	0	63	1	127	5.5%	0.66 [0.03, 16.53]	2011
Eom 2012	8	100	31	348	70.1%	0.89 [0.40, 2.00]	2012
Guan 2012	0	41	0	56		Not estimable	2012
Siani 2012	0	25	0	25		Not estimable	2012
Kim 2013	3	139	1	207	4.3%	4.54 [0.47, 44.14]	2013
Lee 2013	1	50	1	50	5.4%	1.00 [0.06, 16.44]	2013
Jeong 2013	1	122	1	122	5.5%	1.00 [0.06, 16.17]	2013
Total (95%CI)		753		1170	100.0%	1.26 [0.69, 2.30]	
Total events	17		36				

Heterogeneity: $\chi^2 = 3.33$, $df = 7$ ($P = 0.85$); $I^2 = 0\%$

Test for overall effect: $Z = 0.75$ ($P = 0.46$)

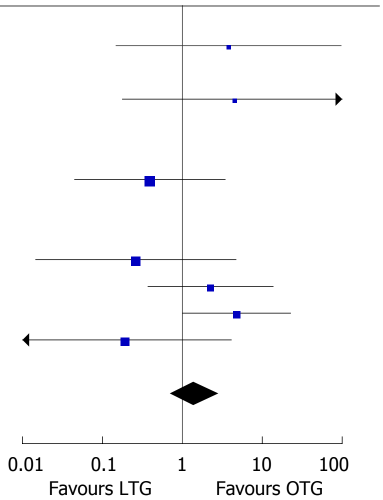


E

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Kim 2008	1	27	0	33	3.3%	3.79 [0.15, 96.92]	2008
Mochiki 2008	0	20	0	18		Not estimable	2008
Sakuramoto 2009	1	30	0	44	3.0%	4.53 [0.18, 114.89]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	1	63	5	127	25.3%	0.39 [0.04, 3.44]	2011
Guan 2012	0	41	0	56		Not estimable	2012
Siani 2012	0	25	0	25		Not estimable	2012
Eom 2012	0	100	6	348	22.5%	0.26 [0.01, 4.69]	2012
Kim 2013	3	139	2	207	12.2%	2.26 [0.37, 13.71]	2013
Jeong 2013	9	122	2	122	14.4%	4.78 [1.01, 22.60]	2013
Lee 2013	0	50	2	50	19.2%	0.19 [0.01, 4.10]	2013
Total (95%CI)		753		1170	100.0%	1.42 [0.70, 2.87]	
Total events	15		17				

Heterogeneity: $\chi^2 = 7.75$, $df = 6$ ($P = 0.26$); $I^2 = 23\%$

Test for overall effect: $Z = 0.98$ ($P = 0.33$)

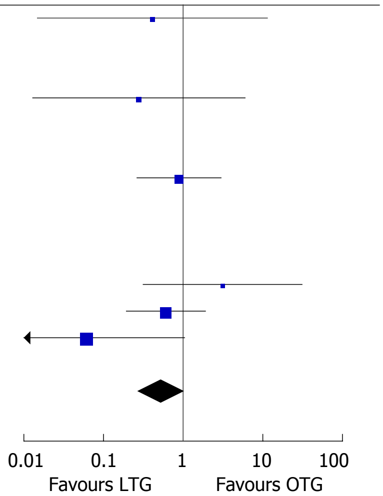


F

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	1	11	4.6%	0.41 [0.01, 11.46]	2005
Kim 2008	0	27	0	33		Not estimable	2008
Mochiki 2008	0	20	0	18		Not estimable	2008
Sakuramoto 2009	0	30	2	44	7.5%	0.28 [0.01, 6.01]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	4	63	9	127	21.0%	0.89 [0.26, 3.01]	2011
Siani 2012	0	25	0	25		Not estimable	2012
Eom 2012	0	100	0	348		Not estimable	2012
Guan 2012	0	41	0	56		Not estimable	2012
Lee 2013	3	50	1	50	3.5%	3.13 [0.31, 31.14]	2013
Jeong 2013	5	122	8	122	28.8%	0.61 [0.19, 1.92]	2013
Kim 2013	0	139	11	207	34.6%	0.06 [0.00, 1.05]	2013
Total (95%CI)		753		1170	100.0%	0.53 [0.28, 1.03]	
Total events	12		32				

Heterogeneity: $\chi^2 = 5.43$, $df = 5$ ($P = 0.37$); $I^2 = 8\%$

Test for overall effect: $Z = 1.88$ ($P = 0.06$)

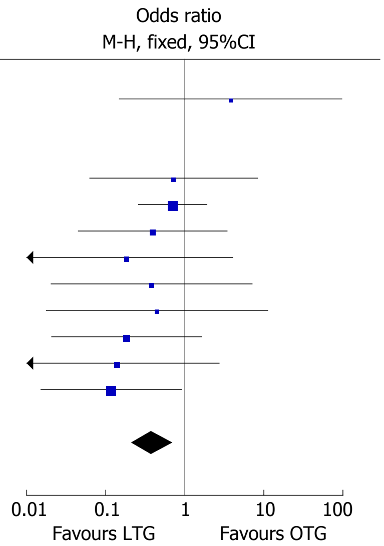


G

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Kim 2008	1	27	0	33	1.1%	3.79 [0.15, 96.92]	2008
Mochiki 2008	0	20	0	18		Not estimable	2008
Kawamura 2009	0	46	0	35		Not estimable	2009
Sakuramoto 2009	1	30	2	44	4.1%	0.72 [0.06, 8.36]	2009
Du 2010	7	82	11	94	24.5%	0.70 [0.26, 1.91]	2010
Kim 2011	1	63	5	127	8.5%	0.39 [0.04, 3.44]	2011
Siani 2012	0	25	2	25	6.4%	0.18 [0.01, 4.04]	2012
Eom 2012	0	100	4	348	5.2%	0.38 [0.02, 7.13]	2012
Guan 2012	0	41	1	56	3.3%	0.45 [0.02, 11.22]	2012
Lee 2013	1	50	5	50	12.8%	0.18 [0.02, 1.63]	2013
Jeong 2013	0	122	3	122	9.1%	0.14 [0.01, 2.73]	2013
Kim 2013	1	139	12	207	25.0%	0.12 [0.02, 0.92]	2013
Total (95%CI)		753		1170	100.0%	0.39 [0.21, 0.72]	
Total events	12		45				

Heterogeneity: χ^2 = 5.95, *df* = 9 (*P* = 0.75); I^2 = 0%

Test for overall effect: *Z* = 3.03 (*P* = 0.002)



H

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	1	11	17.2%	0.41 [0.01, 11.46]	2005
Topal 2008	1	38	1	22	17.4%	0.57 [0.03, 9.55]	2008
Mochiki 2008	0	20	0	18		Not estimable	2008
Kim 2008	0	27	0	33		Not estimable	2008
Sakuramoto 2009	0	30	0	44		Not estimable	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	2	94	32.8%	0.22 [0.01, 4.74]	2010
Kim 2011	0	63	0	127		Not estimable	2011
Guan 2012	0	41	0	56		Not estimable	2012
Eom 2012	1	100	3	348	18.7%	1.16 [0.12, 11.29]	2012
Siani 2012	0	25	0	25		Not estimable	2012
Kim 2013	0	139	0	207		Not estimable	2013
Lee 2013	0	50	0	50		Not estimable	2013
Jeong 2013	2	122	1	122	13.9%	2.02 [0.18, 22.54]	2013
Total (95%CI)		791		1192	100.0%	0.74 [0.24, 2.31]	
Total events	4		8				

Heterogeneity: χ^2 = 1.55, *df* = 4 (*P* = 0.82); I^2 = 0%

Test for overall effect: *Z* = 0.52 (*P* = 0.61)

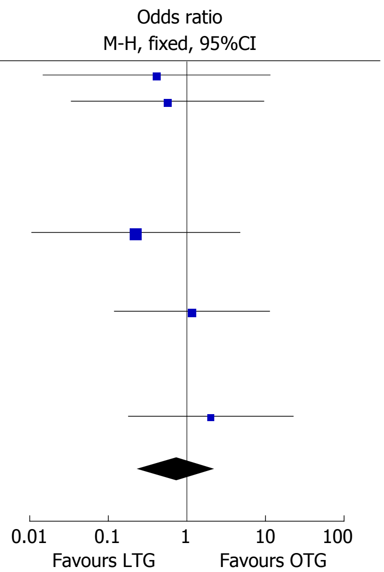


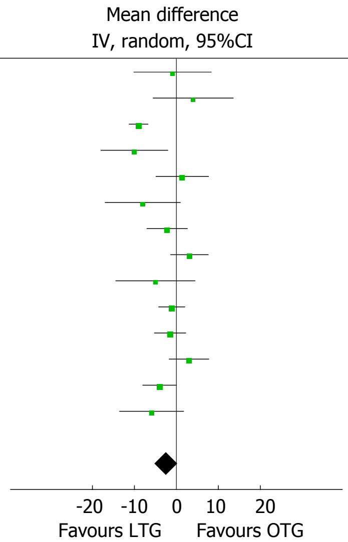
Figure 4 Forest plots illustrating results of postoperative complications in the form of a meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy for gastric cancer. Pooled odds ratio (OR) with 95%CI was calculated using the fixed-effects model. A: Overall complication rate; B: Anastomotic leak; C: Anastomotic Stenosis; D: Ileus; E: Bleeding; F: Abdominal abscess; G: Wound-related complications; H: Mortality. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

A

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Usui 2005	28	15.1	20	28.9	14.3	19	4.8%	-0.90 [-10.13, 8.33]	2005
Dulucq 2005	24	12	8	20	8	11	4.6%	4.00 [-5.57, 13.57]	2005
Mochiki 2008	26	3	20	35	4	18	10.2%	-9.00 [-11.27, -6.73]	2008
Kim 2008	27.2	15.7	27	37.2	15.7	33	5.6%	-10.00 [-17.99, -2.01]	2008
Kawamura 2009	48.5	16.3	46	47.1	12.5	35	6.9%	1.40 [-4.87, 7.67]	2009
Sakuramoto 2009	43.2	17.2	30	51.2	22.1	44	5.0%	-8.00 [-16.97, 0.97]	2009
Du 2010	34.2	13.5	82	36.4	19.1	94	8.1%	-2.20 [-7.04, 2.64]	2010
Kim 2011	38.7	15.7	63	35.6	13.1	127	8.4%	3.10 [-1.40, 7.60]	2011
Siani 2012	35	18	25	40	16	25	4.7%	-5.00 [-14.44, 4.44]	2012
Guan 2012	23.1	8	41	24.2	7.5	56	9.6%	-1.10 [-4.24, 2.04]	2012
Eom 2012	48.3	16.4	100	49.8	18.4	348	9.1%	-1.50 [-5.25, 2.25]	2012
Kim 2013	37	24	139	34	18.8	207	8.2%	3.00 [-1.74, 7.74]	2013
Jeong 2013	42	15	122	46	17	122	8.9%	-4.00 [-8.02, 0.02]	2013
Lee 2013	48.4	18.4	50	54.3	20.5	50	5.9%	-5.90 [-13.54, 1.74]	2013
Total (95%CI)			773			1189	100.0%	-2.49 [-5.18, 0.21]	

Heterogeneity: Tau² = 17.15; χ^2 = 50.76, *df* = 13 (*P* < 0.00001); I^2 = 74%

Test for overall effect: *Z* = 1.81 (*P* =0.07)

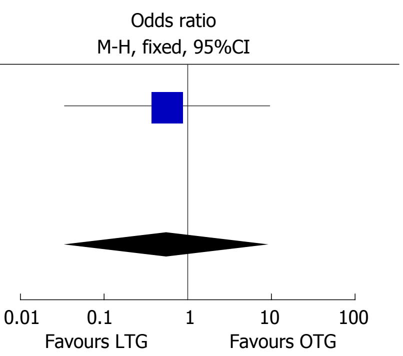


B

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Topal 2008	1	38	1	22	100.0%	0.57 [0.03, 9.55]	2008
Du 2010	0	82	0	94		Not estimable	2010
Guan 2012	0	41	0	56		Not estimable	2012
Kim 2013	0	139	0	207		Not estimable	2013
Total (95%CI)		308		390	100.0%	0.57 [0.03, 9.55]	
Total events	1		1				

Heterogeneity: Not applicable

Test for overall effect: Z = 0.39 (P = 0.69)

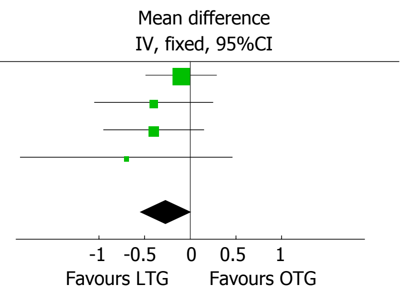


C

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, fixed, 95%CI	Year
Du 2010	3	1.4	82	3.1	1.2	94	50.8%	-0.10 [-0.49, 0.29]	2010
Kim 2011	2.8	2.3	63	3.2	1.8	127	18.2%	-0.40 [-1.05, 0.25]	2011
Eom 2012	4.4	2.4	100	4.8	2.7	348	25.4%	-0.40 [-0.95, 0.15]	2012
Kim 2013	2.3	4.2	139	3	6.8	207	5.7%	-0.70 [-1.86, 0.46]	2013
Total (95%CI)			384			776	100.0%	-0.26 [-0.54, 0.01]	

Heterogeneity: χ^2 = 1.63, df = 3 (P = 0.65); I^2 = 0%

Test for overall effect: Z = 1.88 (P = 0.06)



D

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, fixed, 95%CI	Year
Du 2010	3.1	1.8	82	2.9	0.6	94	78.9%	0.20 [-0.21, 0.61]	2010
Kim 2011	13.4	4.9	63	13.2	4.8	127	6.1%	0.20 [-1.27, 1.67]	2011
Eom 2012	11.4	4.7	100	10.8	5	348	11.7%	0.60 [-0.46, 1.66]	2012
Kim 2013	13.5	10.9	139	11.2	6.2	207	3.3%	2.30 [0.30, 4.30]	2013
Total (95%CI)			384			776	100.0%	0.32 [-0.05, 0.68]	

Heterogeneity: χ^2 = 4.39, df = 3 (P = 0.22); I^2 = 32%

Test for overall effect: Z = 1.71 (P = 0.09)

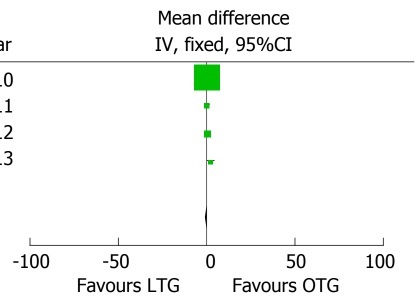


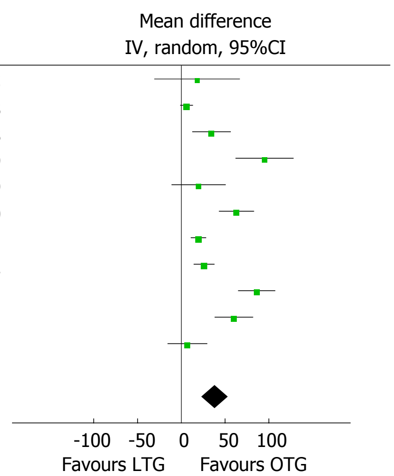
Figure 5 Forest plots illustrating results of oncological outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI were calculated using the fixed or random-effects model. A: No. of resected lymph nodes; B: Positive resection margins; C: Proximal resection margin; D: Distal resection margin. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

A

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	183	48	8	165	60	11	5.5%	18.00 [-30.62, 66.62]	2005
Mochiki 2008	254	10	20	248	12	18	11.0%	6.00 [-1.07, 13.07]	2008
Topal 2008	187	60	38	152.5	25	22	9.3%	34.50 [12.75, 56.25]	2008
Sakuramoto 2009	313	81	30	218	53	44	7.6%	95.00 [62.06, 127.94]	2009
Kawamura 2009	291.9	59.4	46	272.1	76.8	35	7.9%	19.80 [-10.89, 50.49]	2009
Du 2010	275	78	82	212	51	94	9.6%	63.00 [43.22, 82.78]	2010
Kim 2011	150.8	31.2	63	131.2	21.6	127	10.8%	19.60 [11.03, 28.17]	2011
Siani 2012	211	23	25	185	19	25	10.6%	26.00 [14.31, 37.69]	2012
Jeong 2013	289	89	122	203	78	122	9.4%	86.00 [65.00, 107.00]	2013
Lee 2013	258	54	50	198	57	50	9.3%	60.00 [38.24, 81.76]	2013
Kim 2013	144	104.3	139	137	105	207	9.2%	7.00 [-15.48, 29.48]	2013
Total (95%CI)			623			755	100.0%	38.78 [22.81, 54.74]	

Heterogeneity: Tau^2 = 592.67; χ^2 = 105.91, df = 10 (P < 0.00001); I^2 = 91%

Test for overall effect: Z = 4.76 (P < 0.00001)

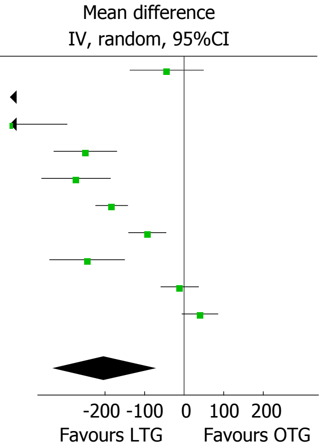


B

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	81	107	8	125	95	11	9.9%	-44.00 [-137.00, 49.00]	2005
Mochiki 2008	229	50	20	758	78	18	10.3%	-529.00 [-571.17, -486.83]	2008
Topal 2008	10	98.8	38	450	337.5	22	9.2%	-440.00 [-584.49, -295.51]	2008
Kawamura 2009	54.9	45.3	46	304.3	237.3	35	10.0%	-249.40 [-329.10, -169.70]	2009
Sakuramoto 2009	134	98	30	407	270	44	9.9%	-273.00 [-360.15, -185.85]	2009
Du 2010	156	112	82	339	162	94	10.3%	-183.00 [-223.74, -142.26]	2010
Kim 2011	179.7	123.8	63	272.7	209.6	127	10.2%	-93.00 [-140.58, -45.42]	2011
Siani 2012	250	150	25	495	190	25	9.8%	-245.00 [-339.89, -150.11]	2012
Lee 2013	167.3	135.2	50	178.4	107	50	10.2%	-11.10 [-58.89, 36.69]	2013
Jeong 2013	249	204	122	209	157	122	10.2%	40.00 [-5.68, 85.68]	2013
Total (95%CI)			484			548	100.0%	-200.47 [-330.87, -70.06]	

Heterogeneity: Tau² = 42687.18; χ^2 = 442.70, *df* = 9 (*P* < 0.00001); I^2 = 98%

Test for overall effect: *Z* = 3.01 (*P* = 0.003)

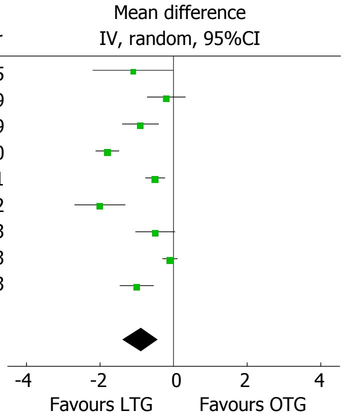


C

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	3.6	1.2	8	4.7	1.2	11	7.7%	-1.10 [-2.19, -0.01]	2005
Kawamura 2009	4.1	1	46	4.3	1.3	35	11.2%	-0.20 [-0.72, 0.32]	2009
Sakuramoto 2009	2.4	1.1	30	3.3	1	44	11.3%	-0.90 [-1.39, -0.41]	2009
Du 2010	3.5	0.8	82	5.3	1.3	94	12.2%	-1.80 [-2.11, -1.49]	2010
Kim 2011	3.3	0.7	63	3.8	0.8	63	12.4%	-0.50 [-0.76, -0.24]	2011
Siani 2012	2.1	0.9	25	4.1	1.5	25	10.2%	-2.00 [-2.69, -1.31]	2012
Lee 2013	4	1.2	50	4.5	1.5	50	11.1%	-0.50 [-1.03, 0.03]	2013
Jeong 2013	2.9	0.8	122	3	0.8	122	12.6%	-0.10 [-0.30, 0.10]	2013
Kim 2013	3	2	139	4	2.3	207	11.5%	-1.00 [-1.46, -0.54]	2013
Total (95%CI)			565			651	100.0%	-0.88 [-1.35, -0.40]	

Heterogeneity: Tau² = 0.46; χ^2 = 103.35, *df* = 8 (*P* < 0.00001); I^2 = 92%

Test for overall effect: *Z* = 3.60 (*P* = 0.0003)

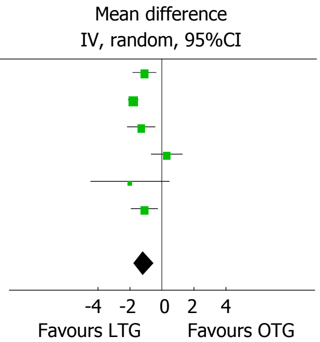


D

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Sakuramoto 2009	4.9	1.1	30	6	2.1	44	19.2%	-1.10 [-1.83, -0.37]	2009
Du 2010	3.5	0.8	82	5.3	1.3	94	24.3%	-1.80 [-2.11, -1.49]	2010
Kim 2011	4.3	1.7	63	5.6	4.4	127	17.4%	-1.30 [-2.17, -0.43]	2011
Jeong 2013	3.9	4.4	122	3.6	3.3	122	16.0%	0.30 [-0.68, 1.28]	2013
Kim 2013	3	12.3	139	5	10	207	5.3%	-2.00 [-4.46, 0.46]	2013
Lee 2013	5	1.7	50	6.1	2.5	50	17.8%	-1.10 [-1.94, -0.26]	2013
Total (95%CI)			486			644	100.0%	-1.13 [-1.76, -0.49]	

Heterogeneity: Tau² = 0.40; χ^2 = 18.90, *df* = 5 (*P* = 0.002); I^2 = 74%

Test for overall effect: *Z* = 3.49 (*P* = 0.0005)

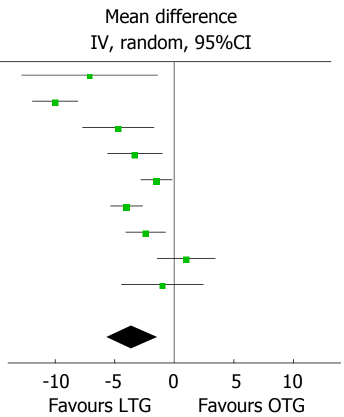


E

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	16.9	3	8	24	9	11	6.8%	-7.10 [-12.81, -1.39]	2005
Mochiki 2008	19	3	20	29	3	18	12.1%	-10.00 [-11.91, -8.09]	2008
Sakuramoto 2009	13.5	2.7	30	18.2	9.6	44	10.5%	-4.70 [-7.70, -1.70]	2009
Kawamura 2009	15.5	3.3	46	18.8	6.3	35	11.6%	-3.30 [-5.59, -1.01]	2009
Kim 2011	8.1	3.8	63	9.6	5.3	127	12.7%	-1.50 [-2.82, -0.18]	2011
Siani 2012	10.5	1.5	25	14.5	3.1	25	12.7%	-4.00 [-5.35, -2.65]	2012
Lee 2013	9.3	4.2	50	11.7	4.3	50	12.4%	-2.40 [-4.07, -0.73]	2013
Jeong 2013	11.8	11.8	122	10.8	7	122	11.4%	1.00 [-1.43, 3.43]	2013
Kim 2013	7	19.3	139	8	9	207	9.9%	-1.00 [-4.43, 2.43]	2013
Total (95%CI)			503			639	100.0%	-3.55 [-5.65, -1.44]	

Heterogeneity: Tau² = 8.64; χ^2 = 72.78, *df* = 8 (*P* < 0.00001); I^2 = 89%

Test for overall effect: *Z* = 3.30 (*P* = 0.0010)

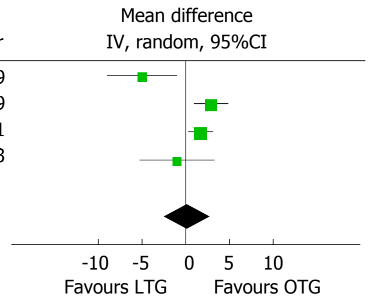


F

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Sakuramoto 2009	6.8	6.4	30	11.8	11	44	19.8%	-5.00 [-8.98, -1.02]	2009
Kawamura 2009	6.9	5.6	46	4	3.2	35	29.6%	2.90 [0.97, 4.83]	2009
Kim 2011	5.3	4.9	63	3.6	3.9	127	32.0%	1.70 [0.31, 3.09]	2011
Kim 2013	3	24.5	139	4	9.3	207	18.6%	-1.00 [-5.27, 3.27]	2013
Total (95%CI)			278			413	100.0%	0.23 [-2.45, 2.91]	

Heterogeneity: Tau² = 5.33; χ^2 = 13.66, *df* = 3 (*P* = 0.003); I^2 = 78%

Test for overall effect: *Z* = 0.17 (*P* = 0.87)

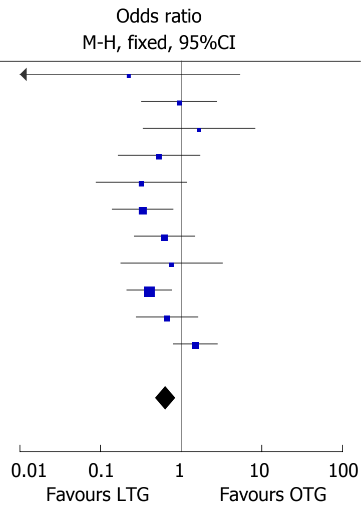


G

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	2	11	1.6%	0.22 [0.01, 5.34]	2005
Topal 2008	15	38	9	22	5.5%	0.94 [0.32, 2.75]	2008
Mochiki 2008	5	20	3	18	1.9%	1.67 [0.34, 8.26]	2008
Sakuramoto 2009	5	30	12	44	6.4%	0.53 [0.17, 1.71]	2009
Kawamura 2009	4	46	8	35	6.6%	0.32 [0.09, 1.17]	2009
Du 2010	8	82	23	94	15.4%	0.33 [0.14, 0.79]	2010
Kim 2011	8	63	24	127	11.0%	0.62 [0.26, 1.48]	2011
Siani 2012	4	25	5	25	3.3%	0.76 [0.18, 3.25]	2012
Kim 2013	14	139	45	207	25.8%	0.40 [0.21, 0.77]	2013
Lee 2013	12	50	16	50	9.7%	0.67 [0.28, 1.62]	2013
Jeong 2013	29	122	21	122	12.7%	1.50 [0.80, 2.81]	2013
Total (95%CI)		623		755	100.0%	0.65 [0.49, 0.86]	
Total events	104		168				

Heterogeneity: χ^2 = 14.72, *df* = 10 (*P* = 0.14); I^2 = 32%

Test for overall effect: *Z* = 3.05 (*P* = 0.002)

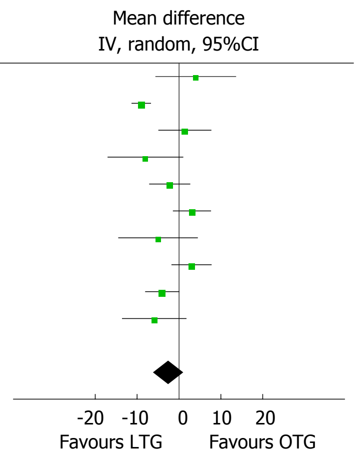


H

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Dulucq 2005	24	12	8	20	8	11	7.1%	4.00 [-5.57, 13.57]	2005
Mochiki 2008	26	3	20	35	4	18	13.3%	-9.00 [-11.27, -6.73]	2008
Kawamura 2009	48.5	16.3	46	47.1	12.5	35	9.9%	1.40 [-4.87, 7.67]	2009
Sakuramoto 2009	43.2	17.2	30	51.2	22.1	44	7.6%	-8.00 [-16.97, 0.97]	2009
Du 2010	34.2	13.5	82	36.4	19.1	94	11.2%	-2.20 [-7.04, 2.64]	2010
Kim 2011	38.7	15.7	63	35.6	13.1	127	11.6%	3.10 [-1.40, 7.60]	2011
Siani 2012	35	18	25	40	16	25	7.2%	-5.00 [-14.44, 4.44]	2012
Kim 2013	37	24	139	34	18.8	207	11.3%	3.00 [-1.74, 7.74]	2013
Jeong 2013	42	15	122	46	17	122	12.0%	-4.00 [-8.02, 0.02]	2013
Lee 2013	48.4	18.4	50	54.3	20.5	50	8.7%	-5.90 [-13.54, 1.74]	2013
Total (95%CI)			585			733	100.0%	-2.28 [-5.93, 1.37]	

Heterogeneity: Tau² = 24.69; χ^2 = 43.65, *df* = 9 (*P* < 0.00001); I^2 = 79%

Test for overall effect: *Z* = 1.23 (*P* = 0.22)

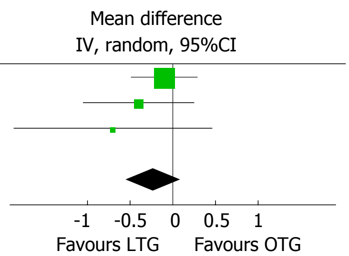


I

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Du 2010	3	1.4	82	3.1	1.2	94	68.0%	-0.10 [-0.49, 0.29]	2010
Kim 2011	2.8	2.3	63	3.2	1.8	127	24.4%	-0.40 [-1.05, 0.25]	2011
Kim 2013	2.3	4.2	139	3	6.8	207	7.6%	-0.70 [-1.86, 0.46]	2013
Total (95%CI)			284			428	100.0%	-0.22 [-0.54, 0.10]	

Heterogeneity: χ^2 = 1.32, *df* = 2 (*P* = 0.52); I^2 = 0%

Test for overall effect: *Z* = 1.34 (*P* = 0.18)

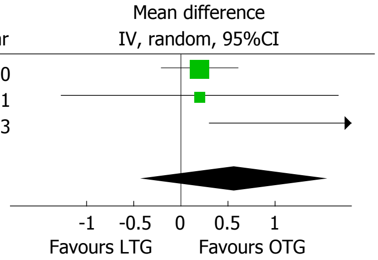


J

Study or subgroup	LTG Mean	LTG SD	LTG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI	Year
Du 2010	3.1	1.8	82	2.9	0.6	94	56.1%	0.20 [-0.21, 0.61]	2010
Kim 2011	13.4	4.9	63	13.2	4.8	127	26.3%	0.20 [-1.27, 1.67]	2011
Kim 2013	13.5	10.9	139	11.2	6.2	207	17.6%	2.30 [0.30, 4.30]	2013
Total (95%CI)			284			428	100.0%	0.57 [-0.42, 1.56]	

Heterogeneity: Tau² = 0.41; χ^2 = 4.08, *df* = 2 (*P* = 0.13); I^2 = 51%

Test for overall effect: *Z* = 1.13 (*P* = 0.26)

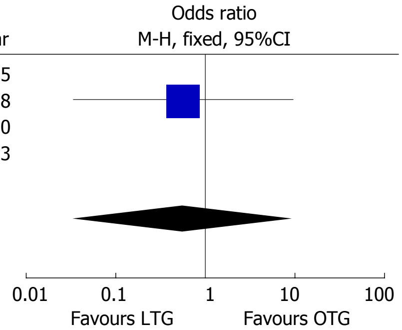


K

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Topal 2008	1	38	1	22	100.0%	0.57 [0.03, 9.55]	2008
Du 2010	0	82	0	94		Not estimable	2010
Kim 2013	0	139	0	207		Not estimable	2013
Total (95%CI)		267		334	100.0%	0.57 [0.03, 9.55]	
Total events	1		1				

Heterogeneity: Not applicable

Test for overall effect: *Z* = 0.39 (*P* =0.69)

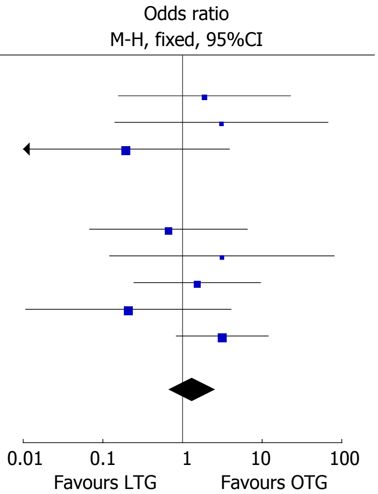


L

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Mochiki 2008	2	20	1	18	6.7%	1.89 [0.16, 22.79]	2008
Topal 2008	2	38	0	22	4.1%	3.08 [0.14, 67.16]	2008
Sakuramoto 2009	0	30	3	44	19.7%	0.19 [0.01, 3.90]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	1	63	3	127	13.8%	0.67 [0.07, 6.54]	2011
Siani 2012	1	25	0	25	3.3%	3.12 [0.12, 80.39]	2012
Lee 2013	3	50	2	50	13.2%	1.53 [0.24, 9.59]	2013
Kim 2013	0	139	3	207	19.7%	0.21 [0.01, 4.09]	2013
Jeong 2013	9	122	3	122	19.5%	3.16 [0.83, 11.97]	2013
Total (95%CI)		623		755	100.0%	1.35 [0.68, 2.66]	
Total events	18		15				

Heterogeneity: χ^2 = 5.67, *df* = 7 (*P* =0.58); I^2 = 0%

Test for overall effect: *Z* = 0.85 (*P* =0.39)

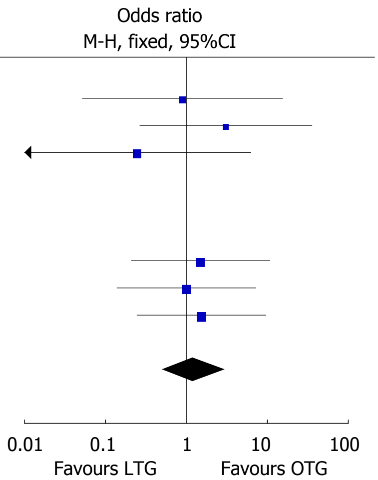


M

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Mochiki 2008	1	20	1	18	11.3%	0.89 [0.05, 15.44]	2008
Kawamura 2009	0	46	1	35	19.0%	0.25 [0.01, 6.26]	2009
Sakuramoto 2009	2	30	1	44	8.5%	3.07 [0.27, 35.49]	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	0	63	0	127		Not estimable	2011
Siani 2012	0	25	0	25		Not estimable	2012
Kim 2013	2	139	2	207	17.9%	1.50 [0.21, 10.75]	2013
Jeong 2013	2	122	2	122	22.2%	1.00 [0.14, 7.22]	2013
Lee 2013	3	50	2	50	21.2%	1.53 [0.24, 9.59]	2013
Total (95%CI)		585		733	100.0%	1.22 [0.51, 2.96]	
Total events	10		9				

Heterogeneity: χ^2 = 1.67, *df* = 5 (*P* = 0.89); I^2 = 0%

Test for overall effect: *Z* = 0.45 (*P* = 0.65)

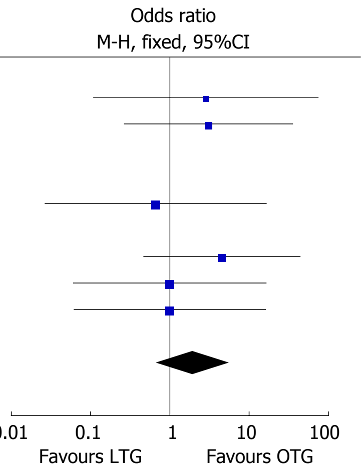


N

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Mochiki 2008	1	20	0	18	9.8%	2.85 [0.11, 74.38]	2008
Sakuramoto 2009	2	30	1	44	15.2%	3.07 [0.27, 35.49]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	0	63	1	127	19.9%	0.66 [0.03, 16.53]	2011
Siani 2012	0	25	0	25		Not estimable	2012
Kim 2013	3	139	1	207	15.7%	4.54 [0.47, 44.14]	2013
Lee 2013	1	50	1	50	19.6%	1.00 [0.06, 16.44]	2013
Jeong 2013	1	122	1	122	19.9%	1.00 [0.06, 16.17]	2013
Total (95%CI)		585		733	100.0%	1.99 [0.69, 5.68]	
Total events	8		5				

Heterogeneity: χ^2 = 1.59, *df* = 5 (*P* = 0.90); I^2 = 0%

Test for overall effect: *Z* = 1.28 (*P* = 0.20)

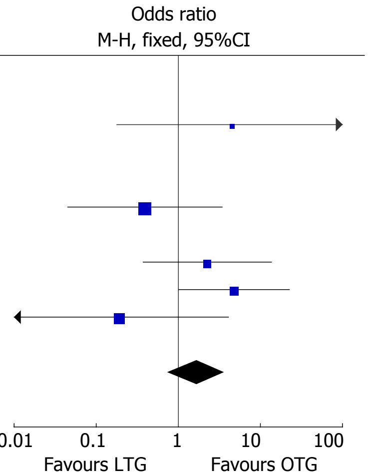


O

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	0	11		Not estimable	2005
Mochiki 2008	0	20	0	18		Not estimable	2008
Sakuramoto 2009	1	30	0	44	4.1%	4.53 [0.18, 114.89]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	1	63	5	127	34.2%	0.39 [0.04, 3.44]	2011
Siani 2012	0	25	0	25		Not estimable	2012
Kim 2013	3	139	2	207	16.5%	2.26 [0.37, 13.71]	2013
Jeong 2013	9	122	2	122	19.4%	4.78 [1.01, 22.60]	2013
Lee 2013	0	50	2	50	25.9%	0.19 [0.01, 4.10]	2013
Total (95%CI)		585		733	100.0%	1.67 [0.76, 3.67]	
Total events	14		11				

Heterogeneity: χ^2 = 5.86, *df* = 4 (*P* = 0.21); I^2 = 32%

Test for overall effect: *Z* = 1.27 (*P* = 0.20)

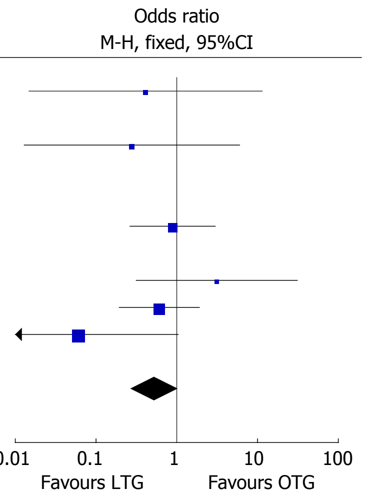


P

Study or subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	Odds ratio M-H, fixed, 95%CI	Year
Dulucq 2005	0	8	1	11	4.6%	0.41 [0.01, 11.46]	2005
Mochiki 2008	0	20	0	18		Not estimable	2008
Sakuramoto 2009	0	30	2	44	7.5%	0.28 [0.01, 6.01]	2009
Kawamura 2009	0	46	0	35		Not estimable	2009
Du 2010	0	82	0	94		Not estimable	2010
Kim 2011	4	63	9	127	21.0%	0.89 [0.26, 3.01]	2011
Siani 2012	0	25	0	25		Not estimable	2012
Lee 2013	3	50	1	50	3.5%	3.13 [0.31, 31.14]	2013
Jeong 2013	5	122	8	122	28.8%	0.61 [0.19, 1.92]	2013
Kim 2013	0	139	11	207	34.6%	0.06 [0.00, 1.05]	2013
Total (95%CI)		585		733	100.0%	0.53 [0.28, 1.03]	
Total events	12		32				

Heterogeneity: χ^2 = 5.43, *df* = 5 (*P* = 0.37); I^2 = 8%

Test for overall effect: *Z* = 1.88 (*P* = 0.06)



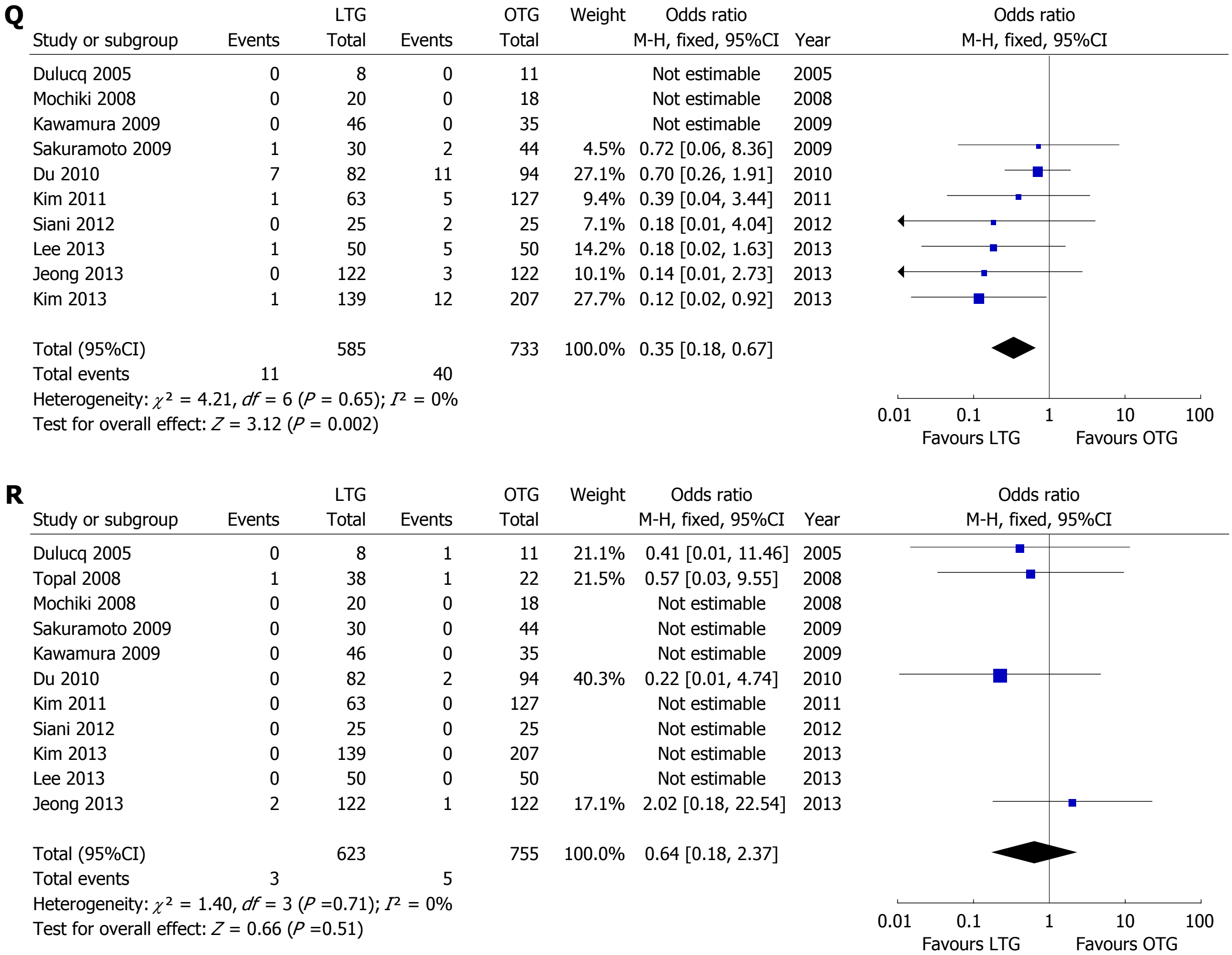


Figure 6 Forest plots illustrating results of all outcomes in the form of a meta-analysis comparing laparoscopic total gastrectomy *vs* open total gastrectomy for gastric cancer. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI were calculated using the fixed or random-effects model. A: Operation time; B: Intraoperative blood loss; C: Time to first flatus; D: Time to first oral intake; E: Hospital stay; F: Analgesics use; G: Postoperative complications; H: No. of resected lymph nodes; I: Proximal resection margin; J: Distal resection margin; K: Positive resection margins; L: Anastomotic leakage; M: Anastomotic Stenosis; N: Ileus; O: Bleeding; P: Abdominal abscess; Q: Wound-related complications; R: Mortality. LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

group. However, there were no significant differences in rate of anastomotic leak, anastomotic stenosis, bleeding, abdominal abscess and postoperative mortality in the two groups. These results indicate that LTG is a safe procedure.

While lymph node metastasis is associated with a poor prognosis in gastric cancer, the extent of lymph node dissection required is open to debate. Many surgeons believe that D1+α or β dissection is adequate for early gastric cancer, and D2 dissection is optimal for advanced gastric cancer, although this remains controversial[41,42]. Surgical removal of at least 15 lymph nodes is advocated in gastric cancer[43]. The mean number of harvested lymph nodes in all included studies was more than 15. The surgical approach did not appear to influence the lymph node yield; however, LTG with extended lymph node dissection may require further refinement of the operative technique and improved instrumentation, and should be performed with caution by surgeons with adequate experience in laparoscopic gastrectomy[29]. Another major concern of laparoscopic resection for gastric cancer is obtaining clear proximal esophageal and distal duodenal margins[17]. Five included studies reported tumor margins, but only one study reported positive resection margins in one patient each in LTG and OTG, respectively; there was no statistically significant difference between the two groups. Our analyses also showed that there was no significant difference in the lengths of the proximal and distal resection margins between the two groups. Seven studies reported data on long-term survival following the two procedures. However, as the duration of follow-up varied between studies, it was difficult to compare them.

Our study has some limitations. Firstly, all the studies included were non-randomized, because of a lack of randomized controlled trials. Secondly, there was significant heterogeneity in the studies with respect to the extent of lymph node dissection, tumor staging and surgical anastomosis techniques. Also, there were differences in the number of patients in the two groups and between studies.

In conclusion, compared with OTG, LTG with regional lymph node dissection for early and advanced gas-

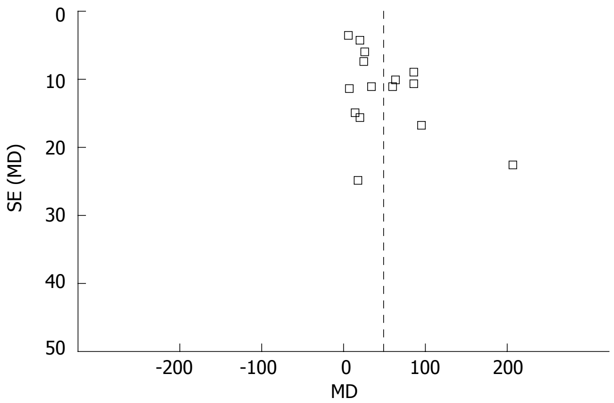


Figure 7 Funnel plot of operation time of all included studies.

tric cancer is safe and effective; with comparable short-term oncological outcomes; lower intraoperative blood loss and overall complication rates; fewer wound-related complications; quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operating time. However, there is a need to develop well-designed, adequately powered, prospective, multicenter, randomized controlled trials, investigating LTG with adequate long-term follow-up, before recommending its wider use in surgical practice.

COMMENTS

Background

Since laparoscopic total gastrectomy (LTG) was first reported in 1999, it has been used increasingly to treat gastric cancer as result of technical advances and improved instrumentation. However, compared with conventional open total gastrectomy (OTG), the safety and efficacy of LTG is not known.

Research frontiers

To conduct a meta-analysis comparing the safety and effectiveness of LTG with OTG in patients with gastric cancer; the available perioperative and oncological outcomes were included in this study.

Innovations and breakthroughs

Based on this meta-analysis, when compared with OTG, LTG for early and advanced gastric cancer is safe and effective; with comparable short-term oncological outcomes; lower intraoperative blood loss and overall complication rates; fewer wound-related complications; quicker recovery of gastrointestinal motility and a shorter hospital stay, albeit with a longer operating time.

Applications

LTG is safe, effective and offers some advantages over OTG in the treatment of early and advanced gastric cancer. However, well-designed prospective multicenter, randomized controlled trials investigating the advantage of LTG with adequate long-term follow-up need to be performed before recommending its wider use in surgical practice.

Peer review

In the future, LTG will be rapidly developed in the field of abdominal minimally invasive surgery. This is a well-written study that clarifies some advantages of LTG in the treatment of patients with early and advanced gastric cancer. This study may be interesting for gastrointestinal surgeons worldwide.

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META-ANALYSIS

Effectiveness of interferon-gamma release assays for differentiating intestinal tuberculosis from Crohn's disease: A meta-analysis

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Abstract

AIM: To investigate the clinical usefulness of interferon-gamma release assays (IGRAs) in the differential diagnosis of intestinal tuberculosis (ITB) from Crohn's disease (CD) by meta-analysis.

METHODS: A systematic search of English language studies was performed. We searched the following databases: Medline, Embase, Web of Science and the Cochrane Library. The Standards for Reporting Diagnostic Accuracy initiative and Quality Assessment for Studies of Diagnostic Accuracy tool were used to assess the methodological quality of the studies. Sensitivity, specificity, and other measures of the accuracy of IGRAs in the differential diagnosis of ITB from CD were pooled and analyzed using random-effects models. Receiver operating characteristic curves were applied to summarize overall test performance. Two reviewers independently judged study eligibility while screening the citations.

RESULTS: Five studies met the inclusion criteria. The average inter-rater agreement between the two reviewers for items in the quality checklist was 0.95. Analysis of IGRAs for the differential diagnosis of ITB from CD produced summary estimates as follows: sensitivity, 0.74 (95%CI: 0.68-0.80); specificity, 0.87 (95%CI: 0.82-0.90); positive likelihood ratio, 5.98 (95%CI: 3.79-9.43); negative likelihood ratio, 0.28 (95%CI: 0.18-0.43); and diagnostic odds ratio, 26.21 (95%CI: 14.15-48.57). The area under the curve was 0.92. The evaluation of publication bias was not significant (P = 0.235).

CONCLUSION: Although IGRAs are not sensitive enough, they provide good specificity for the accurate diagnosis of ITB, which may be helpful in the differential diagnosis of ITB from CD.

Key words: Intestinal tuberculosis; Crohn's disease; Interferon-gamma; Meta-analysis

Core tip: The misdiagnosis rate between Crohn's disease (CD) and intestinal tuberculosis (ITB) is 50%-70%. Interferon-gamma release assays (IGRAs) have been used mainly to identify latent tuberculosis infection in patients in several areas and countries. However, the clinical usefulness of IGRAs in the differential diagnosis of ITB from CD is unknown. This is the first study to investigate the clinical usefulness of IGRAs in the differential diagnosis of ITB from CD by meta-analysis. IGRAs provided good specificity for ITB,

and should be helpful in the differential diagnosis of ITB from CD.

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INTRODUCTION

Tuberculosis (TB) is a major worldwide cause of morbidity and mortality[1,2]. The geography of TB is changing and expanding due to immigration, human immune deficiency virus, immune suppressants, and the development of multidrug-resistant strains of TB[1-5], especially in privileged areas of the world. Intestinal tuberculosis (ITB) is an important extra-pulmonary TB that primarily affects the ileum and colon, causing gastrointestinal symptoms such as diarrhea or abdominal pain. Along with the increased incidence of TB, the incidence of ITB has also increased. Recently, with the emergence of Crohn's disease (CD) in Asian countries[3,6,7], differentiating between ITB and CD is more important than ever. Unfortunately, it is difficult to differentiate ITB from CD due to similar symptoms, and pathologic, radiologic, and endoscopic findings[4,8].

ITB and CD are both chronic granulomatous inflammatory disorders of the intestine[9,10], but have a different pathophysiology, clinical course, and treatment options. ITB could be completely cured if diagnosed early and treated appropriately. CD is not curable and recurs easily. Although several endoscopic and histologic parameters to differentiate these two diseases have been suggested[11,12], a large number of ITB cases are diagnosed by assessing the outcomes of empirical anti-tuberculosis therapy. Moreover, in South Korea, 42%-45% of patients with CD received empirical anti-tuberculosis therapy before they were finally diagnosed with CD[13,14].

A delayed diagnosis of ITB and CD may result in a delay in initiating effective therapy, resulting in a negative economic impact and increased morbidity and mortality. Furthermore, the use of steroids, immune suppressants and biological agents after a presumptive diagnosis of CD, can result in severe and sometimes fatal complications such as systemic dissemination of TB. In recent years, T-cell based interferon-gamma (IFN-γ) release assays (IGRAs) have increasingly been used to replace the traditional tuberculin skin test (TST) as a diagnostic tool for TB. IGRAs have been shown to have superior sensitivity and specificity[15,16]. There are two commercially available methods for IGRAs: the QuantiFERON-TB Gold In-Tube (QFT-G-IT) method and the T-SPOT-TB method. QFT-G-IT uses an enzyme-linked immunosorbent assay to measure antigen-specific production of IFN-γ by circulating T-cells in whole blood being challenged with *Mycobacterium tuberculosis* (MTB)-specific antigens. T-SPOT-TB test is a blood IFN-γ assay measuring the number of activated T-cells by identifying IFN-γ release when stimulated by MTB-specific antigens, including early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). However, whether IGRAs contribute to the differential diagnosis of ITB from CD remains controversial. In the present study, we systematically analyzed and assessed the clinical utility of IGRAs in distinguishing ITB from CD *via* meta-analysis techniques.

MATERIALS AND METHODS

Search strategy and study selection

We searched the following databases: Medline (1980-2013), Embase (1980-2013), Web of Science (1990-2013) and the Cochrane Library. An updated search was carried out in March 2013. The following search terms were used: "intestinal tuberculosis", "Crohn's disease", "interferon-gamma/IFN-γ", "sensitivity", "specificity" and "accuracy". We contacted experts in the specialty and searched the reference lists of primary and review articles. Although no language restrictions were imposed initially, our resources only permitted the review of articles published in the English language for the full text review and final analysis. Conference abstracts and letters were excluded due to unavailable data.

A study was included if it provided both sensitivity (true-positive rate) and specificity (false-positive rate) of IGRAs for the differential diagnosis of ITB from CD, or provided IGRAs values in a dot-plot form which allowed the results to be extracted for individual study subjects. Patients of any age diagnosed with ITB underwent smear or culture of MTB and/or histologic observation of ileum and/or colon tissue, as well as clinical diagnosis, such as response to anti-TB therapy. All patients were diagnosed with CD according to the Japanese diagnostic criteria[17] or the World Health Organization diagnostic criteria[18] based on clinical, endoscopic, radiological and pathological features. In addition, we selected studies which included at least 10 ITB/CD specimens eligible for inclusion in order to reduce selection bias due to a small number of participants. Two reviewers (Chen W and Fan JH) independently judged study eligibility while screening the citations. Disagreements were resolved by consensus.

Data extraction and quality assessment

Two reviewers (Chen W and Fan JH) checked and extracted data independently. The reviewers were blinded to publication details, and disagreements were resolved by consensus. Data retrieved from the reports included participant characteristics, assay methods, sensitivity and specificity data, cutoff values, year of publication, and methodological quality. The value of IGRAs provided in dot plots were measured by placing scalar grids over

Table 1 Summary of the included studies

Study	Country/Area	Patients (*n*)	Assay method	Cutoff	Test results				Quality score	
					TP	FP	FN	TN	STARD	QUADAS
Lee *et al*[28]	South Korea	60	T-SPOT-TB	-	12	8	0	40	16	11
Lei *et al*[29]	China	191	T-SPOT-TB	-	36	5	6	62	18	13
Kim *et al*[30]	South Korea	128	QFT-G-IT	0.35 IU/mL	43	6	21	58	17	12
Li *et al*[31]	China	84	T-SPOT-TB	-	16	16	3	49	17	12
Kim *et al*[32]	South Korea	147	QFT-G-IT	0.35 IU/mL	50	7	25	65	18	13

T-SPOT-TB: An enzyme-linked immunosorbent spot assay; QFT-G-IT: Quanti-FERON-TB Gold In-Tube; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; STARD: Standards for reporting diagnostic accuracy; QUADAS: Quality assessment for studies of diagnostic accuracy.

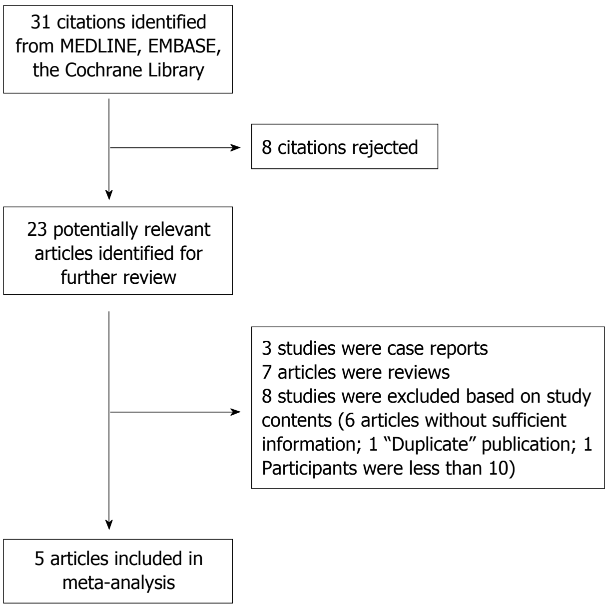

Figure 1 Flowchart of study selection.

the plots, and analyzed using a receiver operating characteristic (ROC) curve for each study (SPSS; Chicago, IL, United States). A summary of each study, including the numbers of true-positive, false-positive, false-negative and true-negative results, is shown in Table 1.

We assessed the methodological quality of studies using guidelines established by the standards for reporting diagnostic accuracy (STARD)[19] initiative and the quality assessment for studies of diagnostic accuracy (QUADAS) tool[20]. In addition, the following study design characteristics were retrieved: (1) cross-sectional design (*vs* case-control design); (2) consecutive or random sampling of patients; (3) blind (single or double) interpretation of determination and reference standard results; and (4) prospective data collection. If primary studies did not show data that met the above criteria, we requested the data from the authors. The "unknown" items were treated as "no" if we did not receive a response from the authors.

Statistical analysis

We used standard methods recommended for meta-analyses of diagnostic test evaluations[21]. Analyses were performed using two professional statistical software programs (STATA, version 11; Stata Corporation, College Station, TX, United States and Meta-DiSc for Windows; XI Cochrane Colloquium; Barcelona, Spain). The following measures of test accuracy were analyzed for each study: sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV), negative predictive value (NPV) and diagnostic odds ratio (DOR).

The analysis was based on a summary ROC (SROC) curve[21]. Sensitivity and specificity as a single test threshold identified for each study were used to plot an SROC curve[22]. A random-effects model was adopted to calculate the average sensitivity, specificity, and other measures across studies[23,24].

The term heterogeneity refers to the degree of variability in results across studies, which was used in relation to meta-analyses. We detected statistically significant heterogeneity with the χ^2 test. To assess the effects of STARD and QUADAS scores on the diagnostic ability of IGRAs, we included them as covariates in the univariate meta-regression analysis (inverse variance weighted). We also analyzed the effects of other covariates on DOR, such as cross-sectional design, consecutive or random sampling of patients, single or double interpretation of determination, reference standard results, and prospective data collection. The relative DOR (RDOR) was calculated according to standard methods to analyze the change in diagnostic precision in the study per unit increase in the covariate[25,26]. Since publication bias is of concern for meta-analyses of diagnostic studies, we tested for the potential presence of this bias with funnel plots and the Egger test[27].

RESULTS

Selection and summary of studies

Five out of 31 publications reporting IFN-γ for the differential diagnosis of ITB from CD were considered to be eligible for inclusion in the analysis[28-32]. Of these 31 publications, 8 citations were rejected, 3 studies were case reports, 7 papers were reviews, and 8 studies were excluded based on study contents (Figure 1). A total of 5 studies including 616 patients were available for analysis, and the clinical characteristics of these studies, along with STARD and QUADAS scores, are outlined in Table 1.

Table 2 Characteristics of the included studies

Ref.	ITB/CD patients (*n*)	Reference standard	Cross-sectional design	Consecutive or random	Blinded design	Prospective
Lee *et al*[28]	12/44	Bac/His or Clin	Unknown	Yes	Unknown	Yes
Lei *et al*[29]	88/103	Bac/His	Unknown	Yes	No	Yes
Kim *et al*[30]	64/64	Bac/His	No	Yes	No	Yes
Li *et al*[31]	19/65	Bac/His or Clin	Yes	Yes	No	Yes
Kim *et al*[32]	75/72	Bac/His or Clin	No	Yes	No	Yes

ITB: Intestinal tuberculosis; CD: Crohn's disease; Bac: Bacteriology; His: Histology; Clin: Clinical course.

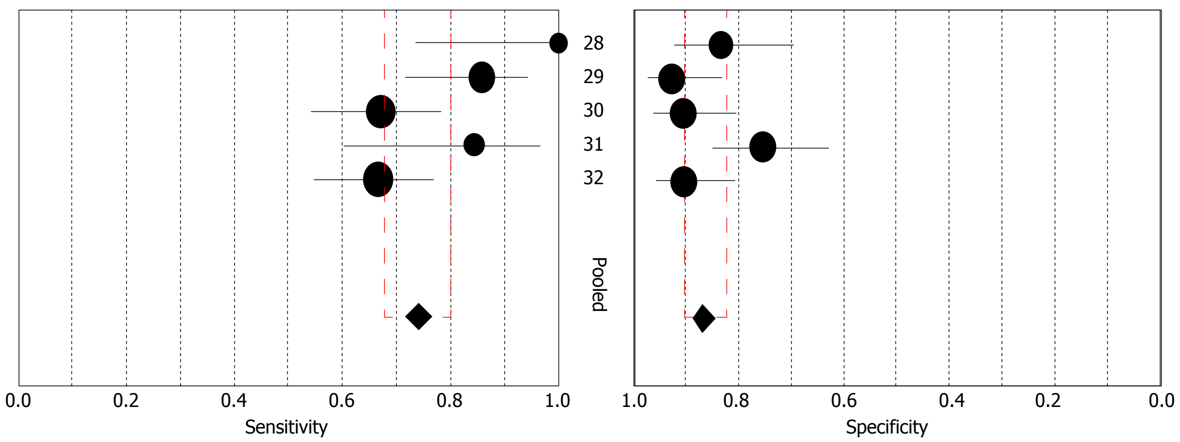

Figure 2 Forest plot of estimates of sensitivity and specificity for interferon-gamma release assays in the differential diagnosis of intestinal tuberculosis from Crohn's disease. Forest plot shows sensitivity and specificity of interferon-gamma release assays for intestinal tuberculosis diagnosis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars indicated 95%CI. Numbers indicate the studies included in the meta-analysis, as cited in the reference list. Pooled estimates for interferon-gamma release assays were as follows: sensitivity, 0.74 (95%CI: 0.68-0.80) and specificity, 0.87 (95%CI: 0.82-0.90).

Quality of reporting and study characteristics

The average inter-rater agreement between the two reviewers for items in the quality checklist was 0.95. All studies were collected from consecutive patients. The average sample size was 112 (range, 60-191) in the included studies. All studies reported that the study design was prospective (Table 2). None of the studies reported blinded interpretation of the IGRAs independent of the reference standard.

Diagnostic accuracy

The sensitivity and specificity of IGRAs in the 5 studies for the differential diagnosis of ITB from CD are shown in the forest plot (Figure 2). Sensitivity of IGRAs for ITB diagnosis ranged from 0.54 to 1.00 (mean, 0.74; 95%CI: 0.68-0.80), while specificity ranged from 0.63 to 0.98 (mean, 0.87; 95%CI: 0.82-0.90). We also noted that PLR was 5.98 (95%CI: 3.79-9.43), NLR was 0.28 (95%CI: 0.18-0.43) and DOR was 26.21 (95%CI: 14.15-48.57). The Chi-square values of sensitivity, specificity, PLR, NLR and DOR were 15.22 (P = 0.0043), 10.55 (P = 0.0322), 9.28 (P = 0.0544), 9.74 (P = 0.0504) and 4.99 (P = 0.2882), respectively, indicating heterogeneity for sensitivity and specificity between studies.

Two methods of IGRAs were used in the included studies in this meta-analysis. One was the T-SPOT-TB test, in which mononuclear cells from blood are used and the number of IFN-γ producing cells responding to antigens such as the ESAT-6 and CFP-10 is reported. The other method of IGRAs was Quanti-FERON-TB Gold In-Tube (QFT-G-IT), which measures T-cell INF-γ production (expressed as pg/mL or IU/mL) in blood in response to a cocktail of ESAT-6, CFP-10 and TB 7.7. The P value following a comparison of overall diagnostic values from T-SPOT-TB and QFT-G-IT was 0.3073. It could not be concluded that the overall accuracy of T-SPOT-TB for the differential diagnosis of ITB from CD was superior or inferior to that of QFT-G-IT.

The SROC plot is different from the traditional ROC plot that explores the effect of varying thresholds on sensitivity and specificity in a single study. In a SROC plot, any of the data points represent a separate study. The SROC curve presents a global summary of test performance and shows the tradeoff between sensitivity and specificity. A graph of the SROC curve for IGRA determination showing true-positive rates and false-positive rates from individual studies is shown in Figure 3. As a global measure of test efficacy we used the Q-value, the intersection point of the SROC curve with a diagonal line from the left upper corner to the right lower corner of the ROC space, which corresponds to the highest common value of sensitivity and specificity for the test. This point represents an overall measure of the discriminatory power of a test. Our data showed that the SROC curve was positioned near the upper left corner and that the maximum joint sensitivity and specificity was 0.87.

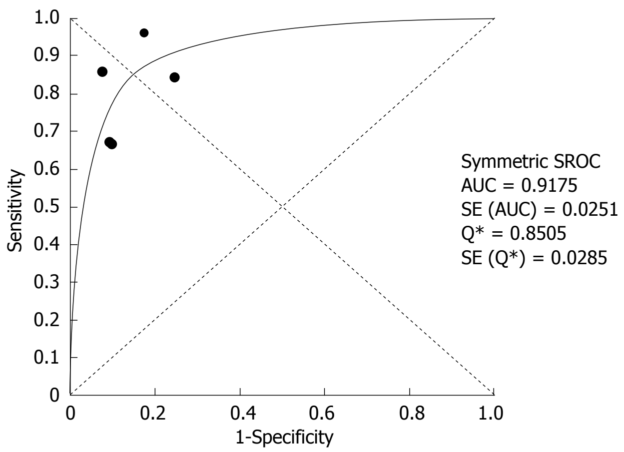

Figure 3 Summary receiver operating characteristic curves for interferon-gamma release assays. Solid circles represent each study included in the meta-analysis. The size of each study is indicated by the size of the solid circle. Summary receiver operating characteristic (SROC) curves summarize the overall diagnostic accuracy.

The area under the curve (AUC) was 0.92. These data indicated that the overall accuracy of IGRAs was not as high as expected.

Multiple regression analysis

By using the STARD guidelines[19], a quality score for each study was compiled on the basis of title and introduction, methods, results and discussion (Table 1). Quality scoring was also carried out using QUADAS[20], in which a score of 1 indicated a fulfilled criterion, 0 if an unclear criterion, and -1 if the criterion was not achieved. These scores were used in the meta-regression analysis to assess the effect of study quality on the RDOR of IGRAs in the differential diagnosis of ITB from CD. All studies were of high quality (STARD score, ⩾ 13; QUADAS score, ⩾ 10) in this review. The differences in the studies with or without blinding, cross-sectional, consecutive/random and prospective designs did not reach statistical significance (P = 0.218), indicating that the study design did not substantially affect the diagnostic accuracy.

Publication bias

Although the Egger test is widely used to evaluate publication bias, it is not useful if less than 10 studies are included. Based on this meta-analysis, which included five articles, we would consider that there was potential for publication bias.

DISCUSSION

The misdiagnosis rate between CD and ITB is 50%-70%[4,5,33,34]. It is important to differentiate between ITB and CD in order to provide effective and prompt therapies due to the increasing incidence of CD and widespread drug-resistant TB[8]. In recent years, methods including TST, MTB culture and acid fast bacilli staining have been used for the detection of TB infection. However, the low sensitivity and specificity and complicated processing of samples has limited the use of these methods[35,36]. New techniques, such as CT enteroclysis, capsule endoscopy, single and double balloon enteroscopy, polymerase chain reaction (PCR) and immunological assays for MTB, have also been used in clinical practice. PCR was associated with high sensitivity, but low specificity[37,38]. Endoscopic and histopathological examinations are also conducted to differentiate between the two disorders[39], but specific and precise criteria are lacking. The T-SPOT-TB test, an IGRA, has mainly been used to identify latent tuberculosis infection in patients in several areas and countries including the United States, Europe and Japan. However, the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD is unknown.

In recent studies, the most popular biomarkers proposed for the diagnosis of TB-related disease were adenosine deaminase and INF-γ[40,41]. The levels of both biomarkers were significantly higher in tuberculous peritonitis than in non-tuberculous peritonitis patients. Both showed relatively high sensitivity and specificity in diagnosing tuberculous peritonitis[42-47]. However, for distinguishing ITB from CD, the present meta-analysis has shown that the mean sensitivity of IRGAs was 0.74, while the mean specificity was 0.87. The maximum joint sensitivity and specificity was 0.85, while the AUC was 0.92, indicating that overall accuracy was relatively high, but not as high as expected.

The DOR is a single indicator of test accuracy that combines the sensitivity and specificity data into a single number[48]. The DOR of a test is the ratio of the odds of positive test results in the patient with disease relative to the odds of positive test results in the patient without disease. The value of DOR ranges from 0 to infinity, and higher values indicate better discriminatory test performance (higher accuracy). A DOR of 1.0 indicates that a test did not discriminate between patients with and those without disease. In the present meta-analysis, the mean DOR was 26.21, indicating that IGRAs may be helpful in the differential diagnosis of ITB from CD.

Since the SROC curve and the DOR are not easy to interpret and use in clinical practice[49], the likelihood ratios are considered to be more clinically meaningful[49]. We also determined both PLR and NLR as measures of diagnostic accuracy. Likelihood ratios of > 10 or < 0.1 generate large and often conclusive shifts from pretest to posttest probability (indicating high accuracy). A PLR value of 5.98 suggests that patients with ITB have an approximately six-fold higher chance of being IFN-γ assay-positive compared with CD patients. This six-fold high probability would be considered not high enough to begin or to continue anti-TB treatment in ITB patients, especially in the absence of any malignant evidence (for clinical purposes). On the other hand, NLR was found to be 0.28 in the present meta-analysis. If the IFN-γ assay result was negative, the probability that this patient has ITB is approximately 28%, which is not low enough to rule out ITB from CD. These data suggest that a negative IFN-γ assay result should not be used alone as a justification to deny or to discontinue anti-TB therapy. The

choice of therapeutic strategy should be based on the results of culture of MTB, morphological observation of capsule endoscopy or single/double balloon enteroscopy, and/or histologic observation of peritoneal tissue, as well as other clinical data, such as response to anti-TB therapy.

The PPV is the proportion of patients with positive test results who are correctly diagnosed, while the NPV is the proportion of patients with negative test results who are correctly diagnosed. The pooled results showed that the PPV for IGRAs was 0.74, suggesting that 26% of positive results would actually be false positives. On the other hand, the NPV for IGRAs was 0.87, indicating a false negative rate of 13%. The relatively high NPV suggests that IGRAs would be acceptable for clinical purposes.

An exploration of the reasons for heterogeneity rather than computation of a single summary measure is an important goal of meta-analysis[50]. In our meta-analysis, both STARD and QUADAS scores were used in the meta-regression analysis to assess the effect of study quality on RDOR. All the studies were of high quality (STARD score of ≥ 13 or QUADAS score of ≥ 10). We found that there was no statistical heterogeneity for sensitivity, specificity, PLR, NLR, and DOR among the studies, which indicated that the differences in the studies with or without blinding, cross-sectional, consecutive/random and prospective designs did not reach statistical significance, and the study design did not substantially affect diagnostic accuracy.

Our meta-analysis has several limitations. Firstly, the exclusion of conference abstracts, letters to the editors, and non-English-language studies might have led to publication bias. Secondly, misclassification bias may have occurred. ITB is not always diagnosed by either histologic or microbiological examination. Some patients were diagnosed with ITB based on the clinical course. This issue regarding accuracy of diagnosis could cause nonrandom misclassification, leading to biased results. Thirdly, all the articles were from Asia, and this may also have led to publication bias. Finally, the number of studies that met the inclusion criteria was not large enough. Multi-center and large blinded randomized controlled trials using IGRAs for ITB diagnosis should be performed.

In conclusion, evidence from the present meta-analysis showed that although IGRAs are not sensitive enough, they did show good specificity for the diagnosis of ITB, which may be helpful in the differential diagnosis of ITB from CD. IFN-γ may be a clinical diagnostic marker for the differential diagnosis of ITB from CD. Currently, the literature focusing on the use of IGRAs in ITB is limited; thus, further large multicenter studies are necessary to substantiate the diagnostic accuracy of IGRAs in patients with ITB or CD.

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COMMENTS

Background

The differential diagnosis of intestinal tuberculosis (ITB) from Crohn's disease (CD) is challenging. The misdiagnosis rate between CD and ITB is 50%-70%. T-cell based interferon-gamma release assays (IGRAs) have increasingly been used as a diagnostic tool in the differential diagnosis of ITB from CD. However, whether IGRAs contribute to accurate ITB diagnosis remains controversial.

Research frontiers

IGRAs have mainly been used to identify latent tuberculosis infection in patients in several areas and countries including the United States, Europe and Japan. However, the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD is unknown.

Innovations and breakthroughs

This is the first time that the clinical usefulness of IGRAs for the differential diagnosis of ITB from CD has been investigated by meta-analysis.

Applications

IGRAs provided good specificity for ITB, and should be helpful in the differential diagnosis of ITB from CD. Interferon-gamma may be a clinical diagnostic marker for the differential diagnosis of ITB from CD.

Terminology

IGRAs: T-cell based interferon-gamma release assays have increasingly been used to replace the traditional tuberculin skin test as a diagnostic tool for tuberculosis. IGRAs have been shown to have superior sensitivity and specificity. ITB: Intestinal tuberculosis is an important extra-pulmonary tuberculosis that primarily affects the ileum and colon, causing gastrointestinal symptoms such as diarrhea or abdominal pain. Standards for reporting diagnostic accuracy and quality assessment for studies of diagnostic accuracy scores: these scores are used in the meta-regression analysis to assess the effect of study quality on relative diagnostic odds ratio.

Peer review

This study is an interesting meta-analysis comment. It provides a new evidence of IGRAs helping differential diagnosis ITB from CD.

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CASE REPORT

Seven synchronous early gastric cancer with 28 lymph nodes metastasis

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Abstract

An 85 year male patient complaining epigastric discomfort was admitted. From the esophagogastroduodenoscopy, three early gastric cancer (EGCa) lesions had been identified and these were diagnosed as adenocarcinoma with poorly differentiated cell type. The patient underwent operation. From the post-operative mapping, however, additional 4 EGCa lesions were found, and the patient was diagnosed with 7 synchronous EGCa. Out of the 7 EGCa lesions, 6 had shown invasion only to the mucosal layer and one had shown invasion into the 1/3 layer of submucosa. In spite of such superficial invasions, 28 of 48 lymph nodes had been identified as metastases. The multiple lesions of EGCa do not increase the risk of lymph node metastasis, but if their differentiations are poor or if they have lympho-vascular invasion, multiple lymph node metastases could incur even if the depth of invasion is limited to the mucosal layer or the upper portion of the submucosal layer.

Key words: Early gastric cancer; Synchronous; Metastasis; Lymph node; Endoscopy

Core tip: Early gastric cancer is often found synchronously in 2 to 3 lesions. However, this case reports on an unprecedented case of 7 lesions of early gastric cancer. Furthermore, this case deserves more attention because 28 out of 48 lymph nodes showed post-operative metastasis, even though there was only 1 invasion to the 1/3 of the submucosal layer and the remaining 6 invading only up to the mucosal layer. This report speaks to the necessity of extra caution in diagnosing multiple synchronous lesions of early gastric cancer with esophagogastro-duodenoscopy.

Seong H, Kim JI, Lee HJ, Kim HJ, Cho HJ, Kim HK, Cheung DY, Kim DJ, Kim W, Kim TJ. Seven synchronous early gastric cancer with 28 lymph nodes metastasis. *World J Gastroenterol* 2013; 19(44): 8141-8145 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i44/8141.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i44.8141

INTRODUCTION

Owing to the recent development of diagnostic technology through esophagogastroduodenoscopy (EGD), the prevalence of early gastric cancer (EGCa) is increasing. Also, reports of multiple synchronous EGCa lesions

are increasing as well. The prevalence of multiple EGCa does not differ between advanced gastric cancer patients as 6%-14%[1] and EGCa patients as 8.3%-17%[2]. Multiple EGCa show a high level of prevalence in elder patients or in male patients, and also when the cancer is well differentiated or invasion is limited to the mucosal layer[3]. And most accessory lesions have been known to occur adjacent to the main lesion with same or even better differentiation[4]. It was found that there was no difference in lymph node metastasis when comparing multiple EGCa with single EGCa in general, but if the invasion depth was deep, the possibility of lymph node metastasis was even higher[3]. If the indications of endoscopic treatment are expanded, since even the surgical treatment tends to orient towards less invasive methods to preserve the normal part of stomach as much as possible, accurate pretreatment diagnosis is important for the multiple lesions of EGCa. In our case, 7 EGCa had been found in an 85 year old male patient and there were multiple lymph node metastases identified post-operatively even though the cancer had shown invasion into the upper portion of the submucosal layer.

CASE REPORT

An 85-year-old male patient complaining of epigastric pain for 3 mo was admitted to our hospital. He had no special medical, family and social history, not to mention cancer, and was found nonspecific in his physical examination and initial laboratory finding. From the results of his physical examination, we found that his blood pressure was 135/87 mmHg, pulse rate was 70 times/min, respiratory rate was 20 times/min and body temperature was 36.5 ℃. The conjunctivae were not pale and no jaundice was observed from the sclerae. There were no palpable lymph nodes from the neck examination, and the auscultation had a normal respiratory sound from the thorax. There was no palpable mass, no tenderness or no rebound tenderness found from the abdominal examination.

From the complete blood count of the laboratory findings, hemoglobin was 12.4 g/dL, white blood cell count was 5670/mm^3 and the platelet count was 212000/mm^3, whereas biochemistry examination revealed, fasting blood glucose as 93 mg/dL, urea nitrogen as 14.5 mg/dL, creatinine as 1.19 mg/dL, aspartate aminotransferase as 30 IU/L and alanine aminotransferase as 22 IU/L, total bilirubin was 0.66 mg/dL, direct bilirubin was 0.22 mg/dL, total protein was 6.7 g/dL and albumin 3.56 g/dL, presenting that all the results were in the normal range. Also, tumor markers such as carcinoembryonic antigen and cancer antigen 19-9 were within normal limit as 2.59 ηg/mL and 11.13 U/mL.

From the EGD, the whole stomach had atrophic mucosal change from antrum to cardia, open type III atrophic gastritis and had no *Helicobacter pylori* infection in Warthin-Starry silver stain. There were findings of a well demarcated erythematous depressed erosion in the sized of 8 mm on the posterior wall of proximal antrum (Figure 1A) and a depressed mucosal lesion with a red colored center, pale boundary and clear margin in the size of 15 mm on the posterior wall of lower body (Figure 1B), as well as a depressed erosion in the sized of 7 mm on the posterior wall of lower body (Figure 1C). The lesions were diagnosed as EGCa IIc and the biopsy revealed an adenocarcinoma, poorly differentiated cell type. Although the lesions occurred in multiple regions showing reddish depression with surrounding white rim, the main lesion was very small and in its early stage, suggesting that they are multiple EGCa rather than metastasis. From the abdominal computerized tomography, the locations of lesions could not be identified. Furthermore there was neither any finding of metastasis to neighboring organs such as the liver or pancreas nor any finding of lymph node enlargement in the neighboring areas.

As the patient had shown three lesions of EGCa, poorly differentiated cell type cell type, we did not perform endoscopic submucosal dissection but instead performed subtotal gastrectomy. After the surgery, we also performed a mapping of the subtotal gastrectomy specimen and were able to diagnose additional lesions (Figure 2). The lesions were flat erosions 6 mm in diameter on the anterior wall of proximal antrum (Figure 1D), depressed mucosal lesion 10 mm in diameter on the anterior wall of lower body (Figure 1E), depressed mucosal lesion 7 mm in diameter on the anterior wall of mid body (Figure 1F) and squamous mucosal lesion in the sized 2 mm on the lesser curvature of mid body (Figure 1G). We had reviewed pictures taken during EGD, but could not find any definite lesion and there were no ulcer findings of each lesions (Figure 1).

In histopathological findings, each lesion was identified as adenocarcinoma, poorly differentiated cell type which was the diffuse type and the growth pattern was infiltrative type in accordance with Lauren's classification. All lesions were composed of poorly differentiated adenocarcinoma. There were no lesions with well or moderate differentiated cancer component and the back ground was atrophic gastritis, marked grade (Figure 3). On the invasion depth, the lesion (Figure 3A, C-G) had invaded into the mucosal layer, while the lesion (Figure 3B), which was the largest, had shown invasion into 1/3 of the submucosal layer, SM1, 1000 μm (Figure 1H and I) (Table 1). There was no perineural invasion but vascular and lymphatic invasion were found from the subtotal gastrectomy specimen. The grade of lymphatic invasion was marked (Figure 1I) and out of the 48 resected lymph nodes, 28 lymph nodes had shown metastasis (Figure 3H). Lymph node metastasis was as follow: 1 (4/4), 3 (4/6), 4 (3/10), 5 (5/6), 6 (12/15), 8a (0/7). According to American Joint Committee on Cancer TNM staging classification for gastric cancer, the final pathologic stage was T1bN3bM0.

DISCUSSION

The diagnosis of EGCa is increasing as the performance

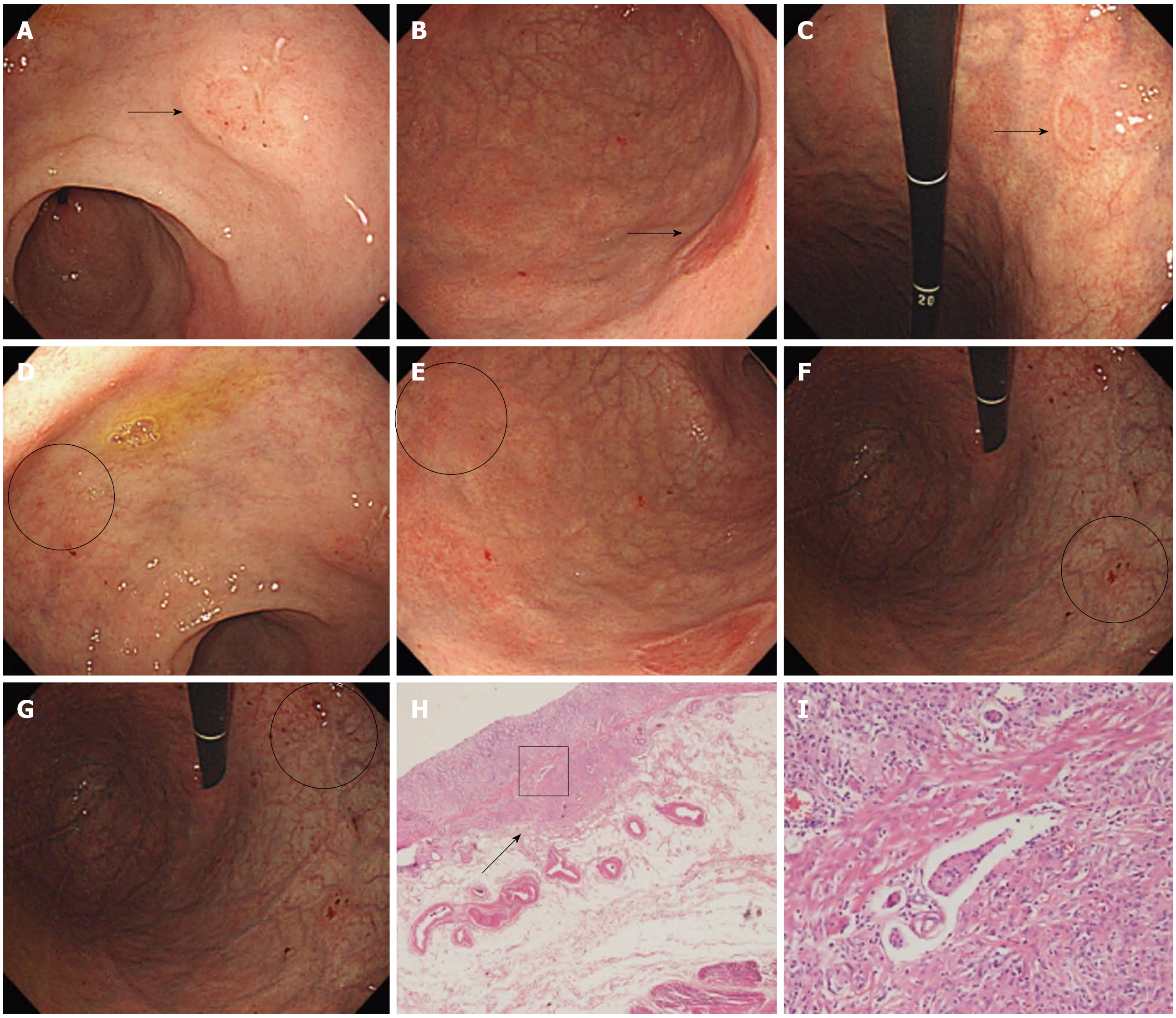

Figure 1 Endoscopic and histologic findings. Multiple early gastric cancer lesions were as follow: A: Raised lesion on the posterior wall of the proximal antrum; B: Erythematous depressive lesion; C: Depressed lesion on the posterior wall of the low body; D: Ill demarcated flat lesion on the anterior wall of the proximal antrum; E, F: Ill demarcated depressed lesion on the anterior wall of the low body (E), on the anterior wall of the mid body (F); G: Ill demarcated flat lesion on the lesser curvature of mid body; H: Adenocarcinoma in lesion B showed invasion into 1/3 of the submucosal layer (arrow) (× 40); I: Lymphatic invasion magnified in quadrangle in H (× 100).

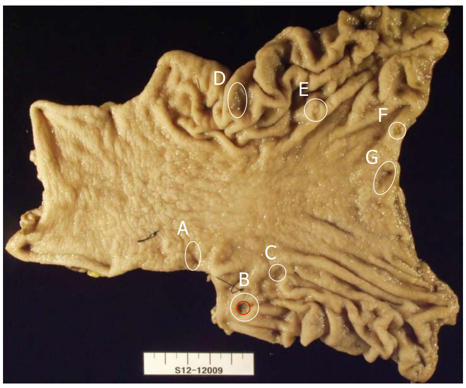

Figure 2 Gross specimen. Three lesions (D-F) are located on the anterior wall, and the other three lesions (A-C) are on the posterior wall. One lesion (G) shows flat early gastric cancer type IIb configuration and is centered at the body, lesser curvature.

of upper endoscopy-related equipments advances and as pathological diagnosis techniques became more developed in recent days. In South Korea, health screening EGD is performed biannually to the entire public nationwide and this led to increase in diagnosis of early stage stomach cancer, thereby leading to increase in diagnosis of multiple EGCa. However, in spite of much effort to find multiple EGCa lesions, the rate of lesions unidentified prior to surgical intervention are as high as 20% to 25%[4].

In the past, gastrectomy was the major treatment of gastric cancer even if multiple gastric cancers were not identified, but because the multiple gastric cancers were included in the resected portion of stomach there was no significant difference in prognosis in some cases. However, in recent days, as endoscopic submucosal dissection is being used as treatment of EGCa, the finding of multiple EGCa is considered important. Moreover, as more

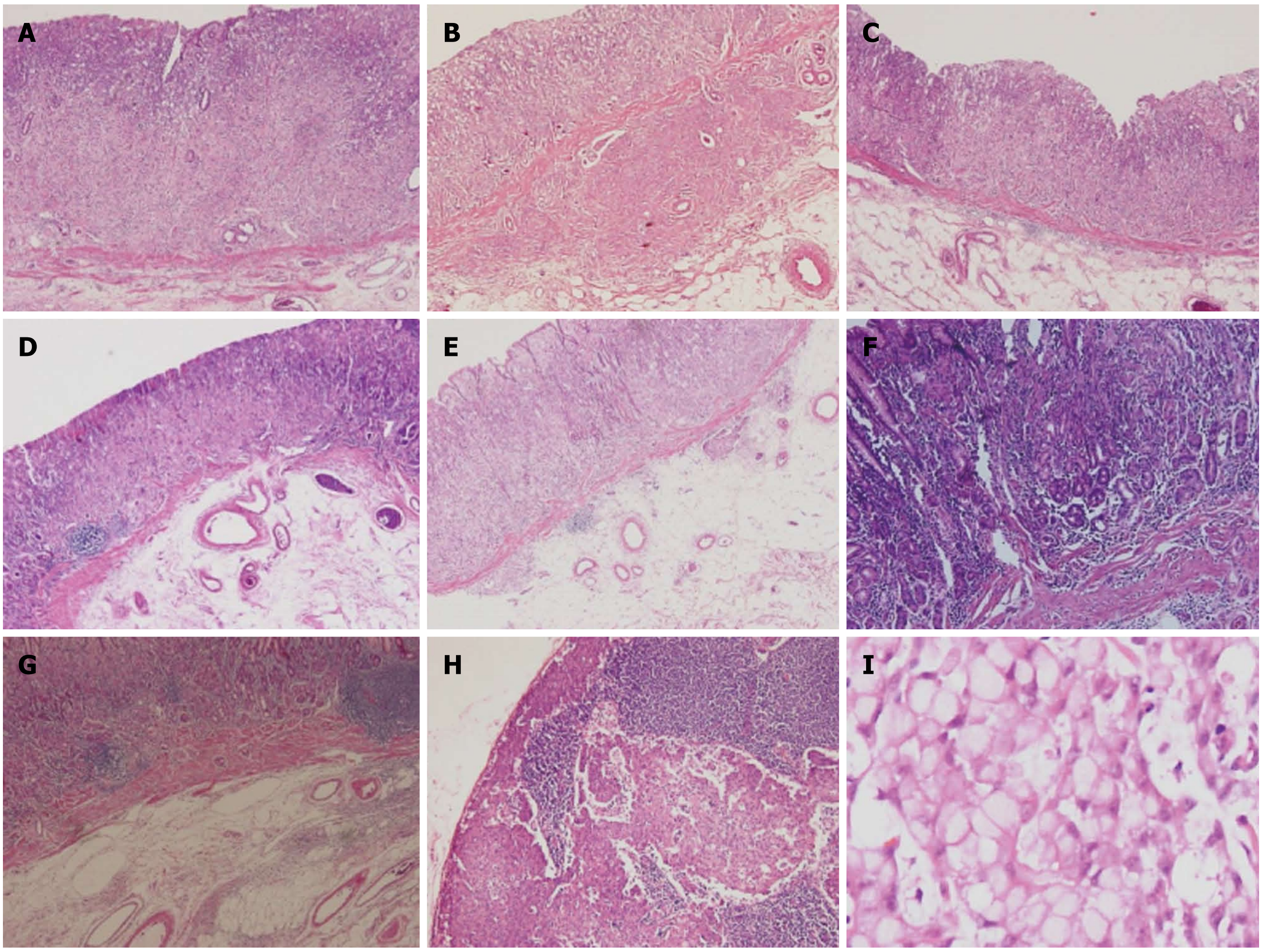

Figure 3 Histopathlogical findings. A-G: Adenocarcinoma, poorly differentiated in each early gastric cancer lesions in Figure 1 (× 40); H: Lymph node metastasis after gastrectomy (× 40); I: Signet ring cell type of biopsy specimen in esophagogastroduodenoscopy (× 100).

Table 1 Cancer type, size and depth of invasion for each lesion of multiple early gastric cancer

	A	B	C	D	E	F	G
Type	Ⅱc	Ⅱc	Ⅱc	Ⅱb	Ⅱc	Ⅱc	Ⅱb
Size (mm^2)	6 × 6	15 × 12	7 × 7	6 × 5	10 × 8	7 × 7	2 × 2
Depth	M	SM1	M	M	M	M	M

M: Mucosa; SM1: A third layer of the submucosa.

non-invasive procedures are preferred for elderly patients for the treatment of EGCa, the importance of finding multiple lesions became more significant.

According to Moertel *et al*[5], diagnosis of multiple EGCa requires evidence of pathological malignancy for each lesion, which should be present at independent locations without the possibility of metastasis from the other organs. Multiple EGCa are more prevalent in male or older patients. In many cases, the area of occurrence is located at middle third portion and lower third portion of the stomach, whereas most accessory lesions incur at adjacent locations to the main lesion, usually the distal part[3,4]. Most multiple EGCa have shapes of the elevated type or flat type rather than the depressed type, and most are associated with well differentiated lesions rather than poorly differentiated lesion, and majority of them show invasion only to the mucosal layer[3,4]. In addition, when there is adenoma or atrophic gastritis, and when the patient has a family history of stomach cancer, multiple EGCa is more likely to be prevalent[1,6].

The prognosis and 5 year survival rate of multiple EGCa as well as single EGCa are both similarly over 90%[7]. The recurrence rate is also similarly 11.2% in both types, and a substantial number of them are considered to be caused by multiple synchronous EGCa which had been overlooked during prior EGD[8]. Therefore, it is important to keep in mind the possible existence of synchronous lesion when establishing plans for examination and treatment.

There are arguments that total gastrectomy should be performed for the treatment of multiple EGCa due to the risk of recurrence but subtotal gastrectomy can also be performed as the treatment of multiple EGCa as they mainly occur at the distal part and not much different in prognosis. In consideration of the post-operative quality of life, even if subtotal gastrectomy is performed when possible with accurate diagnosis, the results are not different from the cases performed with total gastrectomy.

Recently, endoscopic submucosal dissection is increasing trend as the treatment method of EGCa, and lymph node metastasis becomes an important factor in deciding endoscopic therapy over surgical treatment. Other important factors for prediction of lymph node metastasis include presence/absence of ulcer lesion, tumor size, and invasion depth. Due to the increased accuracy of preoperative CT scan and endoscopic ultrasonography, it is easier to find lymph node metastasis, therefore the identification of depth of invasion as major risk factors of lymph node metastasis becomes important[9]. In our case, although the depth of invasion is limited to the mucosal layer or the 1/3 part of the submucosa layer, numerous lymph node metastasis had occurred. Such outcomes were considered to be caused not from the presence of multiple lesions but from the lympho-vascular invasion.

This case was finally diagnosed as a very rare case of EGCa with 7 multiple synchronous EGCa in a male patient of old age. In addition, the patient had shown metastasis of 28 lymph nodes out of 48 resected lymph nodes although its depth of invasion was limited to the mucosal and the 1/3 part of the submucosal layer. Thereby, we report this very rare case with literature review in order to inform the importance of accurate diagnosis on multiple EGCa.

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CASE REPORT

Small cell carcinoma of the liver and biliary tract without jaundice

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Abstract

An 80-year-old woman presenting with chest pain was found to have a large, lobulated soft tissue mass in the liver and nearby tissues on abdominal computed tomography (CT). The tumor had invaded the common hepatic artery and main portal vein. Jaundice developed 4 wk later, at which point, a pancreas and biliary CT scan revealed a large mass in the right lobe of the liver and a hilar duct obstruction, which was found to be a small cell carcinoma. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain without jaundice.

Key words: Extrapulmonary small cell carcinoma; Jaundice; Liver mass; Bile duct mass; Neuroendocrine tumor

Core tip: We report a rare case of small cell carcinoma of the liver and biliary tract. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain because this symptom might indicate the presence of abdominal malignancy. We also explain why previous studies have reported inconsistent immunohistochemical findings in tissues obtained from extrapulmonary small cell carcinomas.

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INTRODUCTION

Most small cell carcinomas occur in the lung, and extrapulmonary small cell carcinoma comprises only 2.5%-4% of all small cell carcinoma cases[1,2]. These malignancies are now considered to be distinct clinicopathological entities from small cell lung cancer, and there is little consensus regarding the optimal treatment strategy in such cases. Extrapulmonary small cell carcinomas are rarely found in the trachea, larynx, thymus, esophagus, stomach, small intestine, colon, prostate, gallbladder, skin, breast, and uterine cervix, and they are even rarer in the liver or biliary tract[3]. To the best of our knowledge, only 10 cases of primary small cell carcinomas of the liver have been reported in the English literature[4-10].

Here, we report the case of a patient with small cell carcinoma of the liver and biliary tract initially presenting with atypical chest pain without jaundice.

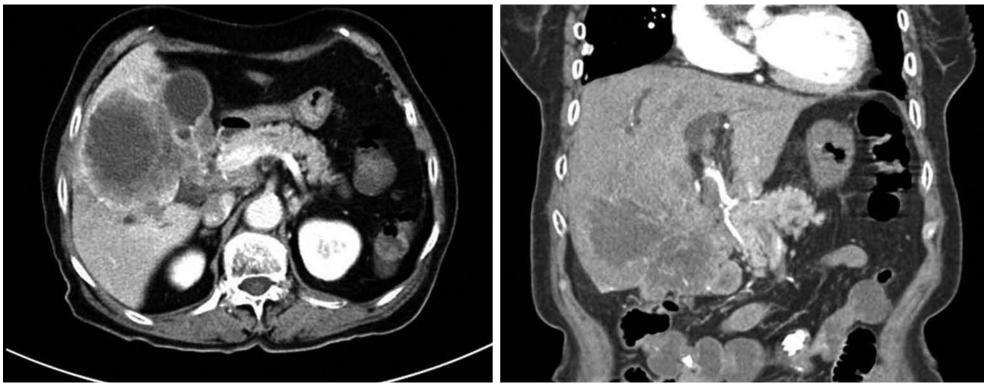

Figure 1 Findings of initial abdominal computed tomography. Computed tomography showed a large mass located in the liver, common hepatic duct, and common bile duct.

CASE REPORT

An 80-year-old woman was admitted to our hospital with a 7-d history of anorexia and chest pain. On admission, she was afebrile, her blood pressure and pulse rate were normal, and she appeared well nourished despite the recent anorexia. During the work-up for chest pain, an electrocardiogram showed a normal sinus rhythm and an echocardiogram showed no significant functional abnormality. The scleras were not icteric. The abdomen was mildly distended, with tenderness in the right upper quadrant. Laboratory studies revealed a white blood cell count of 4500/mm^3 (normal range, 6000-10000/mm^3); hemoglobin, 12.1 g/dL (normal range, 12-16 g/dL); platelet count, 199000/mm^3 (normal range, 130000-450000/mm^3); serum albumin, 3.8 g/dL (normal range, 3.0-5.0 g/dL); aspartate aminotransferase (AST), 101 U/L (normal range, 5-37 U/L); alanine aminotransferase (ALT), 64 U/L (normal range, 5-40 U/L); alkaline phosphatase, 720 U/L (normal range, 39-117 U/L); gamma-guanosine-5'-triphosphate, 215 U/L (normal range, 7-49 U/L); total bilirubin, 0.4 mg/dL (normal range, 0.2-1.2 mg/dL); and proBNP, 170.6 pg/mL (normal range, 0-125 pg/mL). Coagulation profiles were within normal limits. After confirming the absence of a significant cardiac problem, we performed esophagogastroscopy and colonoscopy; however, these investigations also yielded no significant findings, except for that of chronic atrophic gastritis. Abdominal computed tomography (CT) revealed a lobulated soft tissue mass measuring 10.1 cm and located in the liver, common hepatic duct, common bile duct, gall bladder, and hepatoduodenal ligament. The intrahepatic duct was dilated, and the tumor had invaded both the common hepatic artery and main portal vein (Figure 1); multiple enlarged lymph nodes were present near the celiac and common hepatic arteries and in the left gastric and aortocaval spaces. Additional laboratory studies showed that CA19-9 concentration was 12.57 U/mL (normal range, 0-37 U/mL) and α-fetoprotein (AFP) concentration was 2.19 ng/mL (normal range, 0-10.9 ng/mL). Concentrations of carcino-embryonic antigen (CEA) and PIVKAII were not assessed.

These findings led to clinical suspicion of cholangiocarcinoma, but the patient refused to undergo a biopsy and was discharged. However, four weeks later, the patient visited the hospital with jaundice. At this second visit, laboratory analysis of the patient's blood provided the following results: AST, 566 U/L; ALT, 261 U/L; alkaline phosphatase, 6783 U/L; gamma-GTP, 476 U/L; total bilirubin, 25.8 mg/dL; and direct bilirubin, 19.8 mg/dL. A CT scan of the pancreas and biliary duct revealed a large mass in the right lobe of the liver as well as an obstruction of the hilar duct (Figure 2). An ultrasonography-guided gun biopsy was performed, and pathological analysis of the biopsy specimen revealed a tumor consisting of tightly packed nests and diffuse irregularly shaped sheets of cells with areas of necrosis (Figure 3). The tumor cells were of small-to-intermediate size with hyperchromatic, round-to-oval nuclei and scanty, poorly defined cytoplasm. The nuclear chromatin was finely granular, and nucleoli were absent or inconspicuous. Very few cell borders were visible, and there was frequent nuclear molding (Figure 3). The tumor cells were immunoreactive for synaptophysin and CD 56, but negative for hepatocyte-specific antigen (HSA) and thyroid transcription factor (TTF)-1. The Ki-67 index was high (more than 80%), and approximately 5 mitotic cells/10 HPF were observed. Taken together, these findings led us to diagnose small cell carcinoma (Figure 4). On the basis of the World Health Organization 2010 classification for neuroendocrine tumors (NETs)/neuroendocrine carcinomas (NECs), we identified the carcinoma as a grade 3 (G3) NEC (small cell type).

The tumor was unresectable; therefore, palliative chemotherapy was considered to be the best treatment option. However, the patient refused chemotherapy because of her advanced age and poor health. Hence, only supportive care, including percutaneous transhepatic biliary drainage, was provided, and the patient died 8 wk after the diagnosis was confirmed.

DISCUSSION

Extrapulmonary small cell carcinomas represent only 0.1%-0.4% of all cancer cases[11,12]. Most small cell carcinomas arise in the lung or bronchial trees as small cell lung cancers (SCLCs). However, cases of extrapulmonary

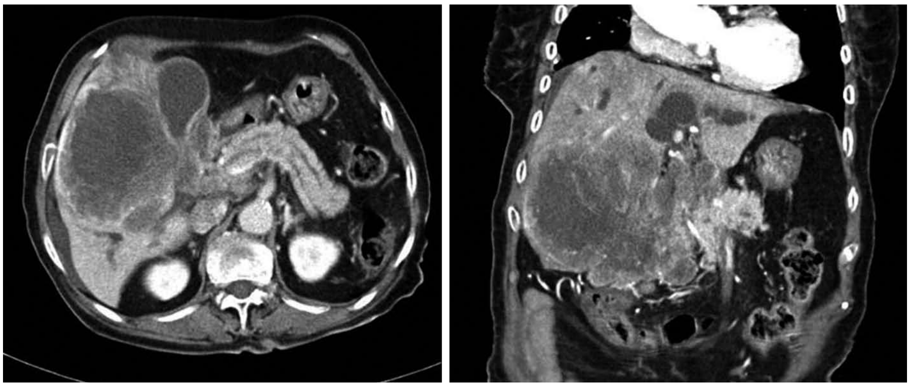

Figure 2 Computed tomography scan obtained before biopsy. One month after the first visit, a computed tomography scan of the pancreas and biliary duct revealed that the large mass in the right lobe of the liver had grown and that there was an obstruction of the hilar duct.

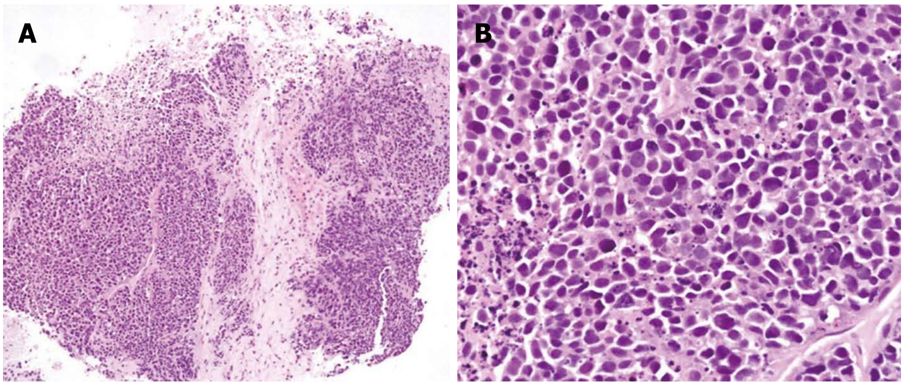

Figure 3 Tumor consisted of tightly packed nests and diffuse, irregularly shaped sheets of cells with necrotic areas. A: The tumor cells were of small-to-intermediate size with hyperchromatic, round-to-oval nuclei and scanty, poorly defined cytoplasm (HE, × 100); B: The nuclear chromatin was finely granular, and nucleoli were absent or inconspicuous. Cell borders were rarely seen, and nuclear molding was common (HE, × 400).

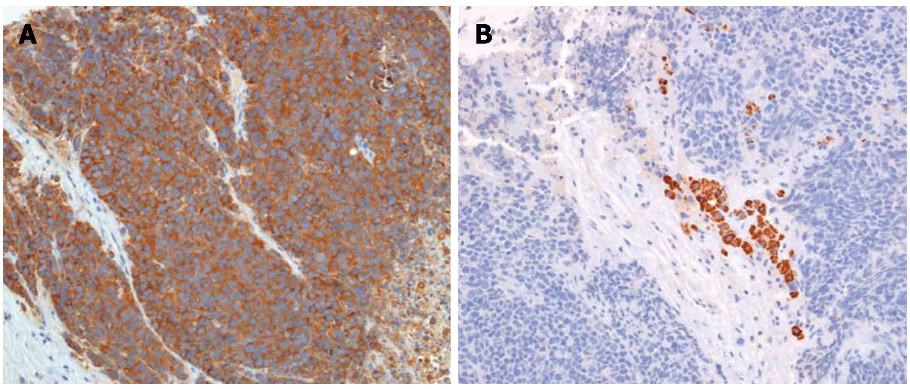

Figure 4 Immunohistochemical findings (× 200). The tumor cells were immunoreactive for synaptophysin (A) and negative for hepatocyte-specific antigen (B).

small cell carcinomas are gradually becoming more common, and there have been reports of small cell carcinomas in both the gall bladder[13] and pancreas[14]. A few studies have reported small cell NEC of the ampulla of Vater[15,16]. However, reports of small cell carcinoma of the liver or common bile duct, as in the present case, are extremely rare.

Differential diagnosis is important to exclude pulmonary small cell carcinoma. In most cases, a finding of occult extrapulmonary small cell carcinoma is subsequently found to be a distant metastasis from an undetected small cell lung cancer. To exclude this possibility, chest radiography and CT, and/or bronchoscopic examination with appropriate biopsies and sputum cytology are required. In addition, growing evidence suggests that positron emission tomography (PET) is also sufficiently sensitive to rule out SCLC[17-19]. In the present case, results of sputum cytology were negative for malignancy, and there was no evidence of lung cancer on chest CT, making bronchoscopy unnecessary.

Extrapulmonary small cell carcinomas shows structural features of both primitive epithelial and neuroendocrine differentiation[17]. Neuroendocrine carcinoma arises from embryonic neural crest cells, which migrate to the bronchopulmonary system and gastrointestinal tracts during development. However, these cells do not usually migrate to the liver and bile duct; this may be the reason for the rare occurrence of neuroendocrine tumors of the liver and bile duct[1,20].

Small cell carcinoma can be distinguished from other carcinomas or lymphomas that have cells of a similar size. Under light microscopy, the diameter of small cell carcinoma cells is generally 2 or 3 times greater than that of a small lymphocyte. In addition, small cell carcinoma cells are spindle-like, have a fusiform or polygonal shape, and tend to grow in sheets or ribbon- or rosette-like patterns. Extensive necrosis and high mitotic rates are also typical features that differentiate small cell carcinoma from atypical carcinoid tumors[5].

Furthermore, several distinct immunohistochemical features associated with extrapulmonary small cell carcinoma are potentially important in distinguishing it from hepatic metastasis of small cell lung carcinoma[21-24]; unfortunately, the results of previous studies are somewhat inconsistent in this respect. Ryu *et al*[4] reported an 8 cm-sized small cell carcinoma of the liver that was positive for CD56/C-kit/synaptophysin and negative for TTF-1. Zanconati *et al*[7] studied 3 cases and found that all of the tumors were positive for AE1/AE3, CK8, CK18, CK19, and NSE and negative for S-100/CEA, and that 2 of them were AFP positive. Frazier *et al*[25] reported a case of small cell carcinoma of the liver in which the patient underwent hepatic segmentectomy and adjuvant etoposide/cisplatin therapy. The tumor in this case was positive for CD56/NSE/c-kit/synaptophysin/mixed CK/EMA and negative for CK7, CK8, CK19, CK20, AFP, CEA, vimentin, and desmin/TTF-1/AFP. In the present case, the tumor was positive for both CK and synaptophysin, weakly positive for CD56, and negative for HSA. These tumor cells originate from multipotent stem cells that can differentiate into various cell types; this may explain the frequent coexistence of mixed tumors with various immunohistochemical features.

Small cell carcinoma frequently shows distant metastasis and consequently has a poor prognosis. Correspondingly, patients with small cell lung cancer generally have poor long-term survival. However, some cases of good long-term survival have been reported in patients with extrapulmonary small cell carcinoma. Little is known about the survival of patients with small cell carcinoma of the liver; generally, the clinical course of this condition is not well described, and reports of patient survival vary. Zanconati *et al*[7] reported two cases in which the patients died soon after diagnosis, and treatment could not be initiated. In contrast, Sengoz *et al*[6] reported two cases of extrapulmonary small-cell carcinoma of the liver, in which one patient survived for 13 mo after chemotherapy and the other survived 67 mo after receiving right hemihepatectomy. Choi *et al*[10] reported the case of a patient who survived more than 18 mo after receiving treatment with oral etoposide alone.

It is often not possible to effectively treat small cell carcinoma of the biliary system with surgical resection alone. As an alternative, Hazama *et al*[26] performed surgical resection after neoadjuvant chemotherapy for small cell carcinoma of the common bile duct, and Okamura *et al*[27] suggested that multimodality treatment including neoadjuvant chemotherapy, surgical resection, and adjuvant chemotherapy improves survival of patients with small cell carcinoma of the biliary system. The generally accepted optimal treatment for extrapulmonary small cell carcinoma is appropriate surgical resection followed by adjuvant chemotherapy. However, Levenson asserted that there is no survival benefit from using surgery to treat either small cell lung cancer or extrapulmonary small cell carcinoma. This may be because the most important prognostic factor is the extent of disease at diagnosis, when most patients with extrapulmonary small cell carcinoma already have occult metastasis[1].

In the present case, we could not administer palliative chemotherapy because of the patient's advanced age and very poor performance status. Although there is no established standard treatment for extrapulmonary small cell carcinoma, chemotherapy should be tried if the patient can tolerate it, because this malignancy is often chemosensitive[28]. The recommended chemotherapy regimen for extrapulmonary small cell carcinoma is the same as that for small cell lung cancer[10]. Furthermore, for patients who are able to undergo surgical resection, platinum-based adjuvant chemotherapy is also advisable, because it can reduce the chance of systemic recurrence[29-31].

In previous reports of primary small cell carcinoma of the liver or bile duct, patients usually presented with jaundice, a palpable mass, or abdominal discomfort as their first symptom. Despite its rarity, liver and bile duct small cell carcinoma should be considered in the differential diagnosis of atypical chest pain as this symptom might indicate the presence of an abdominal malignancy.

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CASE REPORT

Malignant paraganglioma of the rectum: The first case report and a review of the literature

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Abstract

Paragangliomas typically develop in the extra-adrenal sites along the sympathetic and/or the parasympathetic chain. Occasionally, the tumors may arise in some exotic sites, including the head and neck region and the urogenital tract. Paraganglioma presenting as a primary rectal neoplasm has not been well described in the literature. Here, we report the first case of malignant paraganglioma arising in the rectum of a 37-year-old male. He presented to the clinic because of hematochezia with tenesmus. The anorectal digital examination and colonoscopic examination revealed a polypoid mass of the rectum, measuring approximately 4 cm in diameter. The overall morphology and immunophenotype were consistent with a typical paraganglioma. However, the tumor exhibited features suggestive of malignant potential, including local extension into adjacent adipose tissue, nuclear pleomorphism, confluent tumor necrosis, vascular invasion and metastases to regional lymph nodes. In conclusion, we present the first case of rectal malignant paraganglioma. Due to the unexpected occurrence in this region, malignant paraganglioma may be misdiagnosed as other tumors with overlapping features; in particular, a neuroendocrine tumor of epithelial origin. Because of the differences in treatment, separating paraganglioma from its mimics is imperative. Combination of morphology with judicious immunohistochemical study is helpful in obtaining the correct diagnosis.

Key words: Rectum; Paraganglioma; Malignancy

Core tip: We report a rare case of malignant paraganglioma arising in the rectum of a 37-year-old male. To the best of our knowledge, the current case represents the first case of malignant paraganglioma arising in the rectum. Due to the unexpected occurrence in this region, rectal paraganglioma may be misdiagnosed as other common types of tumors with overlapping features; in particular, a neuroendocrine tumor of epithelial origin. Because of the differences in treatment, separating paraganglioma from its mimics is imperative.

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INTRODUCTION

Paragangliomas are rare but well-described non-epithelial neuroendocrine tumors that typically develop in the extra-adrenal sites along the sympathetic and/or the parasympathetic chain[1]. Occasionally, the tumors may arise in some exotic sites where normal paraganglia are not well documented. The majority of these unusual tumors have been described preferentially in the head and neck region and the urogenital tract[2]. With the exception of gangliocytic paraganglioma, paraganglioma is extremely rare in the gastrointestinal tract. Of note, the hitherto reported gas-

trointestinal paragangliomas are exclusively limited to the stomach[3-7]. To the best of our knowledge, paraganglioma has not been well described in the rectum. Due to the unexpected occurrence in this region, rectal paraganglioma may be misdiagnosed as other common types of rectal tumors with morphological overlap. Because the treatments vary, separation of rectal paraganglioma from its mimics, in particular neuroendocrine tumors of epithelial origin, is imperative. In this study, we report a case of malignant paraganglioma presenting as a primary rectal neoplasm to broaden the clinical and morphological spectrum.

CASE REPORT

A 37-year-old male presented to the clinic because of hematochezia. The symptom had lasted for 8 mo and was accompanied intermittently with tenesmus. On anorectal digital examination, a round firm mass was identified on the posterior wall of the rectum. Colonoscopic examination revealed a polypoid mass, measuring approximately 4 cm in diameter. Clinically, the mass was suspected to be a rectal carcinoma. A biopsy was performed and was interpreted as a low-grade neuroendocrine tumor. After the admission, laparoscopic radical rectectomy was performed. The postoperative course was uneventful. The patient received no adjunctive therapy after surgery and is well at 9-mo follow-up.

Pathologic studies and findings

Hematoxylin and eosin-stained sections were reviewed. Immunohistochemical study was performed on 4-mm thick unstained sections of formalin-fixed paraffin-embedded tissue using the standard EnVision technique. The primary antibodies used in the study included antibodies against Chromogranin A (dilution 1:200), synaptophysin (dilution 1:100), neuron-specific enolase (dilution 1:100), CD56 (dilution 1:50), S100 protein (dilution 1:300), pancytokeratin (dilution 1:100), cytokeratin 8/18 (dilution 1:50), epithelial membrane antigen (dilution 1:200), CD34 (dilution 1:50), Human Melanoma Black 45 (dilution 1:60), alpha smooth muscle actin (dilution 1:400), desmin (dilution 1:500), CD117 (dilution 1:100), and discovered on GIST-1 (DOG1) (dilution 1:100). Heat-induced epitope retrieval was performed using a pressure cooker. Appropriate positive controls were run simultaneously throughout the process.

The resected specimen consisted of a segment of rectum measuring 11 cm in length. A polypoid mass was observed protruding into the intestinal cavity, measuring 4.0 cm × 4.0 cm × 1.5 cm in size. On the cut section, the tumor was red-brownish in color and fleshy in consistency, involving the full thickness of the intestinal wall with local extension into the adjacent adipose tissue.

Histologically, the tumor was composed of sheets or organoid nests of large polygonal cells surrounded by a rich network of delicate arborizing vasculature, generating a characteristic "zellballen" (Figure 1A and B). The polygonal cells contained copious eosinophilic to amphiphilic granular cytoplasm, with round to oval nuclei and prominent nucleoli. Although focal nuclear pleomorphism was present (Figure 1C), mitotic activity was relatively low (1-2/50 high power fields). Confluent tumor necrosis and vascular invasion were observed (Figure 1D and E). In addition, the tumor cells metastasized to regional lymph nodes (Figure 1F).

Immunohistochemically, the large polygonal cells exhibited diffuse and strong expression of chromogranin A (Figure 2A), synaptophysin, CD56, neuron-specific enolase and vimentin. In areas with distinct organoid structure, the staining of S100 protein highlighted the presence of slender sustentacular cells located at the periphery of the tumor nests (Figure 2B). However, in areas with more diffuse architecture, the sustentacular cells were difficult to identify. The tumor cells were negative for all of the epithelial, melanocytic, myogenic and Cajal cell markers tested in this study. The Ki67 index was approximately 20% (Figure 2C). The endothelial markers of CD34 and CD31 outlined the rich vascular network (Figure 2D).

DISCUSSION

Paragangliomas have been rarely reported in the gastrointestinal tract. Approximately 12 tumors have been described in this region, most of which were located in the stomach, with only one tumor occurring in the rectum[3-8]. Of note, the only reported rectal paraganglioma was included in a study focusing on a statistical analysis and was only quoted as an anatomic location. Insufficient data were provided in that case, either clinically or pathologically.

In the current study, we present the clinical and pathological features of a malignant paraganglioma occurring in a 37-year-old male who presented with nonspecific symptoms. Clinically, the lesion was suspected as a rectal carcinoma based on anorectal digital findings and rectoscopical examination. Due to the striking organoid structure and strong positivity for neuroendocrine markers, the biopsy specimen was initially interpreted as a low-grade neuroendocrine tumor of epithelial origin, formerly known as a carcinoid tumor. The final diagnosis of paraganglioma was established on the postoperative specimen, which provided enough sections for comprehensive review. The negativity for epithelial marker and presence of slender sustentacular cells allowed the distinction from an epithelial neuroendocrine neoplasm. Other tumors that may enter into the differential diagnosis include neoplasms with perivascular epithelioid cell differentiation (PEComas), alveolar soft part sarcoma (ASPS) and, rarely, gastrointestinal stromal tumor (GIST) of epithelioid subtype. Like paragangliomas, both PEComas and ASPS may exhibit an organoid or nesting pattern surrounded by thin-walled vessels. By immunohistochemistry, PEComas typically express melanocytic and sometimes express myogenic markers, whereas ASPS is characterized by nuclear staining of TFE3 with no expression of neuroendocrine antibodies. The application

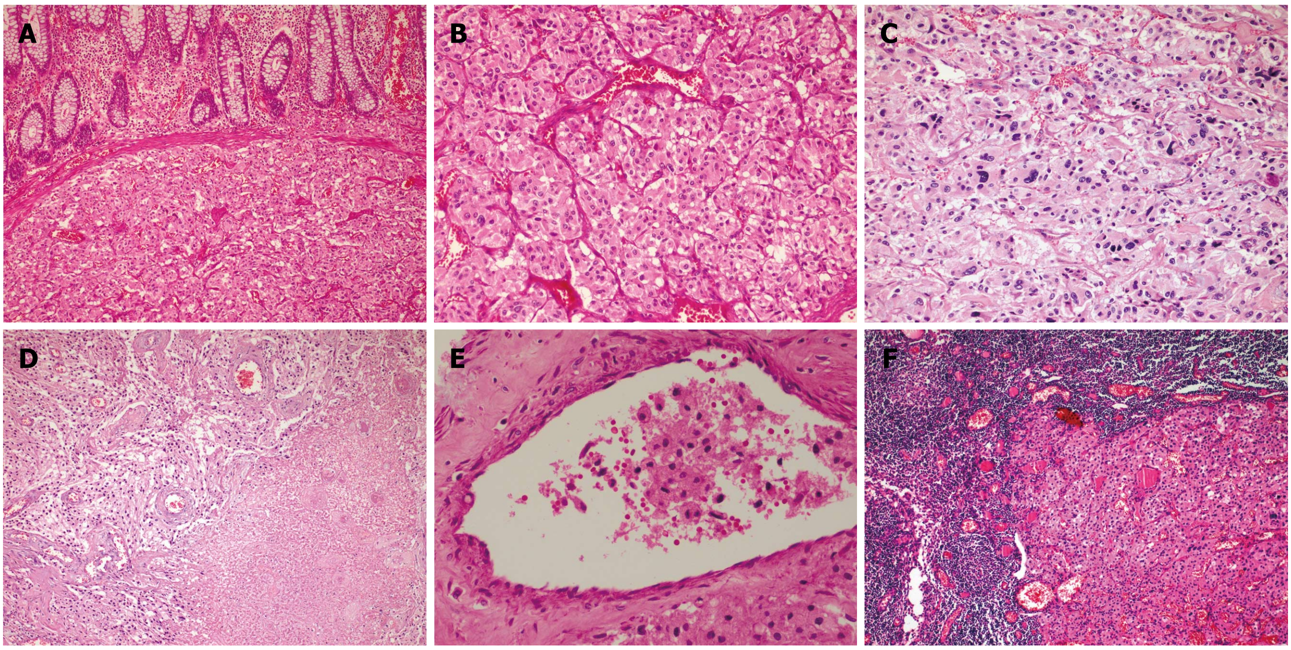

Figure 1 Histological features. A-B: The tumor was composed of sheets or organoid nests of large polygonal cells surrounded by a rich network of delicate arborizing vasculature, generating a characteristic "zellballen" (A: HE, × 100; B: HE, × 400); C: Focal nuclear pleomorphism (HE, × 400); D: Confluent tumor necrosis (HE, × 100); E: Vascular invasion (HE, × 400); F: Metastases of lymph nodes (HE, × 100).

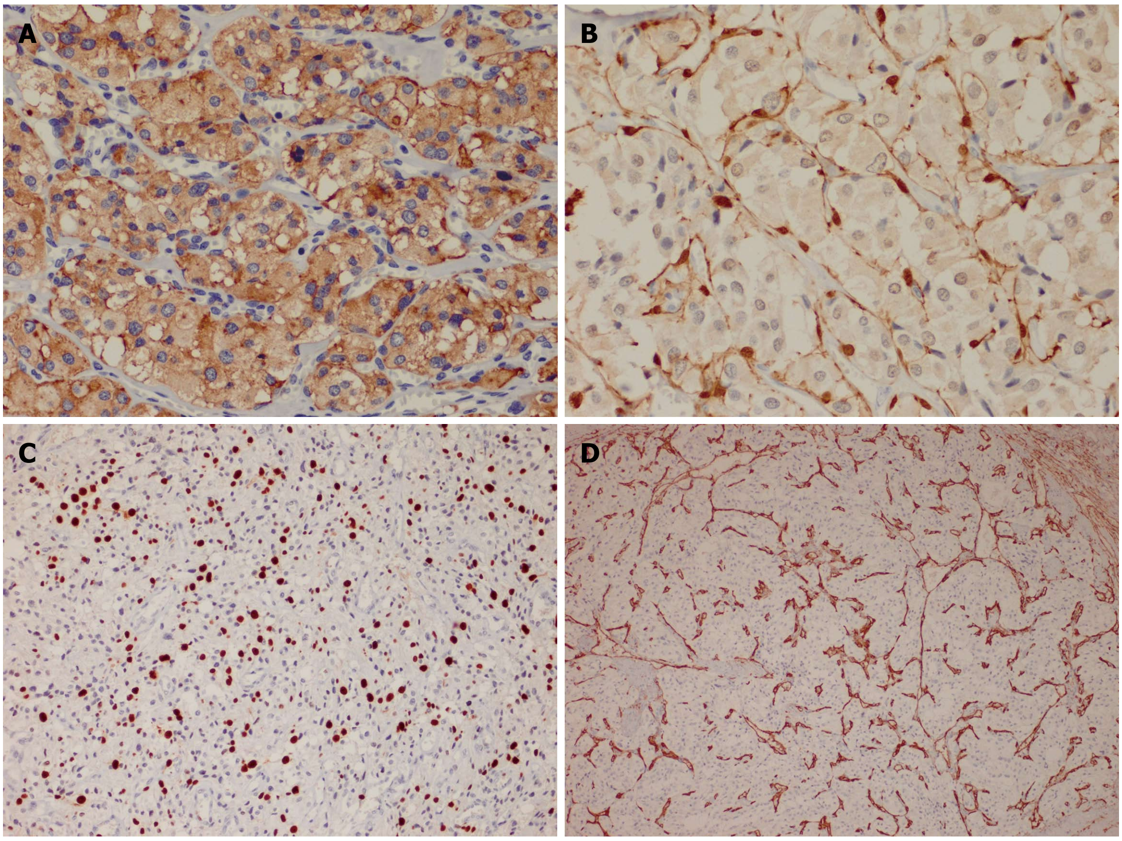

Figure 2 Immunohistochemistry. A: The tumor cells exhibited diffuse and strong expression of chromogranin A; B: S100 protein highlighted the presence of slender sustentacular cells located at the periphery of the tumor nests; C: The Ki67 index was approximately 20%; D: CD34 outlined the rich vascular network.

of Cajal cell markers, namely CD117 and DOG1, will facilitate the distinction from an epithelioid GIST.

Malignant paraganglioma accounts for 14%-50% of all paragangliomas in some large series[9,10]. Only three cases of primary malignant paraganglioma have been reported in the gastrointestinal tract, all of which occurred

in the stomach[4-6]. To the best of our knowledge, rectal malignant paraganglioma has not been described thus far. The current case represents the first case of malignant paraganglioma arising in the rectum.

The diagnosis of malignancy in a paraganglioma is based principally on the aggressive behavior of the tumor. According to the World Health Organization classification of tumors of the endocrine system, the reliable diagnostic criteria of malignant paraganglioma refer to the presence of metastasis or tumor spread in sites normally devoid of chromaffin tissue[11]. Although not considered definitive, several morphological features are believed to be correlated with malignant potential. These features include large size of the tumor (> 5 cm), prominent nuclear pleomorphism, increased mitotic activity, presence of confluent tumor necrosis, diffuse growth pattern with a lack of sustentacular cells, extension to adjacent tissues or structures, vascular invasion and high Ki67 index[11,12]. It is worth noting that none of these features are able to identify a malignant tumor alone. As an alternative approach, some scoring systems have been proposed in the evaluation of malignant potential. One of the most utilized scoring systems is the "Pheochromocytoma of the Adrenal gland Scales Score (PASS)"[13]. A PASS score > 6 is highly suggestive of potential aggressive biological behavior. The current case exhibited local extension into the adjacent adipose tissue, focal nuclear atypia, confluent tumor necrosis, vascular invasion, high Ki67 index and metastases to regional lymph nodes, justifying a diagnosis of malignant paraganglioma.

Recently, an increasing number of studies have focused on identifying molecular markers or biomarkers that can reliably predict malignant potential of the paraganglioma. Several markers, including telomerase, telomerase associated protein, heat shock protein 90, SNAIL and miR-483-5p, also have been found to be closely related to the malignant potential of paraganglioma[10,14]. On the other hand, molecular genetic detection is only gradually being applied to paraganglioma. It has been demonstrated that malignant paraganglioma is strongly associated with SDHB mutations[15]. Despite these recent advances, it remains difficult to reliably predict the outcome of any given patient.

With regard to the prognosis, the 5-year survival rate in malignant paraganglioma is approximately 30%-50%[10]. The majority metastasize to regional lymph nodes, followed by bone, liver and lung[11,14]. At present, there is no optimal therapy for malignant paraganglioma. For the past few years, molecular targeted therapy has been increasingly applied in the therapeutic protocol of malignant paraganglioma. Some targeted agents, such as hypoxia inducible factor-1 a inhibitors, mammalian target of rapamycin inhibitors (everolimus), and receptor tyrosine kinase inhibitors (sunitinib), have been attempted with promising effectiveness[14]. Nevertheless, more clinical trials are needed. The patient in the current study received no adjunctive chemotherapy or radiotherapy. He remains well 9 mo after the surgery and continues to be closely monitored.

In summary, we present the first case of rectal malignant paraganglioma. Because of its rarity in the rectum, malignant paraganglioma may be misdiagnosed as other tumors with overlapping features. Because of the differences in treatment, separation of malignant paraganglioma from its mimics, in particular from neuroendocrine carcinoma, is imperative. Combination of morphology with judicious immunohistochemical study is helpful in obtaining the correct diagnosis.

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CASE REPORT

Ileal conduit stomal variceal bleeding managed by endovascular embolization

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Abstract

Ileal conduit stomal varices are rare, and may result in bleeding. The standard treatment modality for management of this type of hemorrhage has not been established. We present the case of a 70-year-old woman with progressive ileal conduit stomal variceal bleeding which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach. In conclusion, transjugular transhepatic endovascular embolization is a good choice in patients with ileal conduit stomal variceal bleeding who have failed conservative therapy.

Key words: Ectopic variceal bleeding; Ileal conduit; Stomal bleeding; Cirrhosis; Hemostasis; Transjugular transhepatic embolization

Core tip: Ileal conduit stomal varices are very rare, and may result in refractory bleeding. We present the case of a 70-year-old woman with progressive ileal conduit stomal variceal bleeding which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach.

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INTRODUCTION

Varices are a common complication of liver cirrhosis with portal hypertension. Typically, they are found in the gastro-esophageal region. Ectopic varices are rare, and can arise along the entire gastrointestinal tract, including the duodenum, jejunum, ileum, colon and rectum, but seldom at the umbilicus, in the peritoneum and stomas[1,2]. Ectopic varices can present with hemorrhage, accounting for up to 5% of all variceal bleeding[1]. However, due to the difficulty in their diagnosis and treatment, the mortality secondary to their initial bleeding is up to 40%[3]. Currently, reports on ectopic variceal bleeding are mostly located in the gut, especially in the duodenum and rectum. Variceal bleeding from a stoma, especially from an ileal conduit stoma, has rarely been reported[4]. Here, we present a case of ectopic variceal bleeding from an ileal conduit stoma (Figure 1) which was successfully managed by endovascular embolization *via* the transjugular transhepatic approach.

CASE REPORT

A 70-year-old woman, who had undergone cystectomy and an ileal conduit due to interstitial cystitis two years before, presented with chronic bleeding from the ileal

Figure 1 The bluish ectopic varices at the ileal conduit stoma (black arrow).

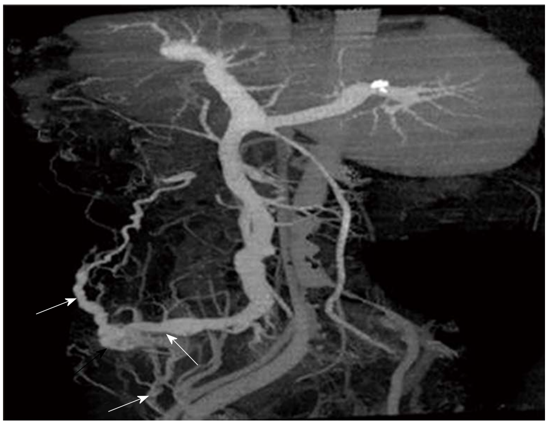

Figure 2 Three-dimensional reconstruction showed the ectopic varices (black arrow) at the ileal conduit stoma fed by the superior mesenteric vein (white arrow) and communicated to the paraumbilical vein (white arrow) and femoral vein (white arrow).

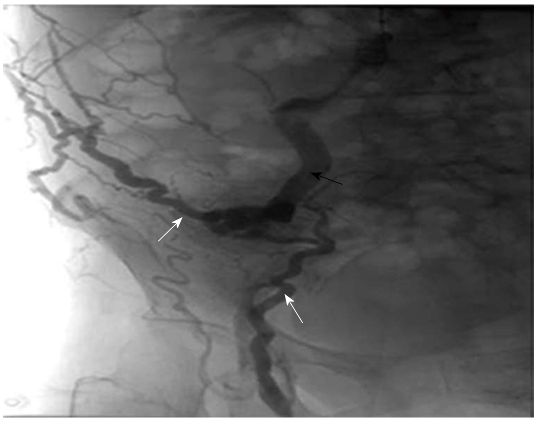

Figure 3 Opacification showed the varices fed by the superior mesenteric vein (black arrow) and communicated to the paraumbilical vein and femoral vein (white arrows).

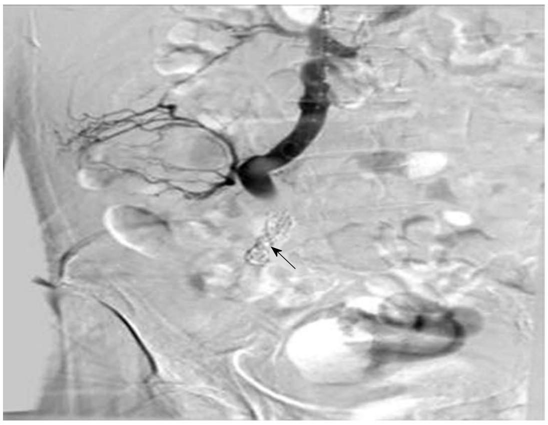

Figure 4 Opacification showed disappearance of the ileal conduit stoma varices and colis (black arrow) in the ectopic varices.

conduit stoma after the operation. The patient also had drug-induced liver cirrhosis and a history of two episodes of hepatic encephalopathy. The bleeding was first considered to be wound bleeding and was treated with homeostatic drugs. However, the hemorrhage persisted, and an enhanced multislice computed tomography (CT) scan and 3-dimensional (3D) reconstruction imaging showed ectopic varices fed by the superior mesenteric vein (SMV) and communicated to the paraumbilical vein and femoral vein at the ileal conduit ostomy (Figure 2). The hemorrhage could be paused by local compression. Two weeks previously, with the bleeding worsening, local compression and vasoactive therapy (Octreotide 50 mg/h) failed to achieve hemostasis. A wound resuture was then performed, however, the result was disappointing. Three days before, massive hemorrhage had occurred and the patient developed hemorrhagic shock (systolic blood pressure 88 mmHg, heart rate 103/min) and became severely anemia (hemoglobin 57 g/L, red blood cell 2.14×10^{12}/L). The platelets, prothrombin time and international normalized ratio were 78×10^{9}/L, 18.6 s, and 1.18, respectively. Considering her hepatic function [Child-Pugh B (9 score)], abundant ascites and her history of recurrent hepatic encephalopathy, an emergency transjugular transhepatic embolization was planned. Portal venography and peripheral superior mesenteric venography demonstrated varices arising from the SMV with retrograde flow toward the stoma and communicated to the paraumbilical vein and femoral vein (Figure 3). The portal-pressure gradient measured during surgery was 18 cmH_2O. Ectopic varices embolization with a stainless steel coil was then performed. The portal-pressure gradient measured after the operation was 19 cmH_2O, and postoperative opacification showed that the varices had disappeared (Figure 4). The patient had no complications following the procedure and received conservative medical therapy with Propranolol after the operation. Follow-up at 4 mo showed no focal bleeding.

DISCUSSION

Reports on the hemorrhage of ectopic varices are limited, and most are of digestive tract bleeding or umbilical vein bleeding[5-9]. Abdominal intestinal ostomy may result in the formation of collateral vessels at the stoma, such as the development of ileostomy stomal varices after ileal conduit ostomy and ileostomy in several case

reports[4,10,11]. The patient in this study developed ileal conduit stomal variceal bleeding. Due to persistent hemorrhage, a contrast enhanced multislice CT scan and 3D reconstruction imaging were performed which revealed ectopic varices fed by the SMV and communicated to the paraumbilical vein and femoral vein at the stoma. The correct diagnosis of variceal bleeding with portal hypertension was made.

The standard therapy for ectopic variceal bleeding has not yet been determined, and a recent review[12] recommended that management should include medical conservative therapy, endoscopic therapy, interventional therapy, surgical shunt or liver transplantation. Similar to variceal bleeding from the esophageal-gastric region, sclerotherapy or band ligation of the varices are theoretically feasible. However, because of potential necrosis and perforation following sclerotherapy, and a high risk of massive hemorrhage following sloughing of the occluded varices after band ligation, and persistence of portal hypertension, the results of this treatment modality were disappointing[13], especially in stomal variceal bleeding[11]. Our patient was treated with ectopic varices suture ligation, however, bleeding did not stop. As transjugular intrahepatic porto-systemic shunt (TIPS) alone or in combination with variceal embolization has demonstrated effectiveness for the hemostasis of ectopic variceal bleeding in patients with portal hypertension in some studies[14,15], it seemed appropriate that our patient should be treated with this modality. However, the current common understanding of TIPS shunt creation for hemostasis is that it increases the incidence of hepatic encephalopathy and damages liver function. Our patient had a history of recurrent hepatic encephalopathy, thus, the TIPS shunt was not applicable in this patient. Surgical shunts have been shown to be effective in preventing hemorrhage recurrence, but are associated with mortality ranging from 1% to 50%, and many patients are not healthy enough to endure the operation[11]. Our patient had hemorrhagic shock, therefore it was clearly unwise to choose this treatment modality. Thus, embolization of the ectopic varices was the best choice for this patient. Usually, varices embolization can be managed *via* the percutaneous transhepatic[16-18] or transjugular transhepatic route[4]. Hemoperitoneum is the most common complication of percutaneous transhepatic embolization[18], other complications include bile leak, liver trauma, and portal thrombosis[17]. As the patient had abundant ascites and we had successfully performed thousands of TIPS procedures, endovascular embolization of the stomal varices *via* the transjugular transhepatic route for hemostasis was the appropriate therapy in this case. The varices were occluded after the procedure and no complications occurred during the procedure. No focal bleeding was observed after four months of follow-up.

In conclusion, although rare, when a patient with ileal conduit stoma presents with persistent stomal bleeding, ectopic variceal bleeding from the ileal conduit should be considered. Endovascular embolization *via* the transjugular transhepatic approach is a reasonable choice in patients with stomal variceal bleeding which failed conservative therapy and local resuture, especially in an emergency situation.

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LETTERS TO THE EDITOR

Hydroxycitric acid does not promote inflammation or liver toxicity

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Abstract

Garcinia cambogia extract (GC) with its active component consisting of hydroxycitric acid (HCA) is widely utilized for weight loss. Various HCA salts are available, including calcium, magnesium, potassium and mixtures of these. Experimentally, these salts exhibit different properties with some, but not all, improving glucose tolerance and blood pressure. Recently, obesity-prone C57BL/6J mice were fed a high-fat diet (HFD, 45 kcal% fat) with or without GC (1%, w/w) for 16 wk. The active arm reduced visceral fat, adipocyte size and serum glucose, yet purportedly also exhibited hepatic collagen accumulation, lipid peroxidation and increased mRNA levels of genes related to oxidative stress. The latter findings are at odds with a large body of animal and human studies that have been conducted on the safety and efficacy of HCA. This literature shows HCA to be protective against the liver toxicity associated with ethanol and dexamethasone administration, and to maintain serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase at near normal levels. In both animal and clinical literature, elevated intakes of HCA *per se* have not led to signs of inflammation or hepatotoxicity. The compound has been found to reduce markers of inflammation in brain, intestines, kidney and serum.

Key words: Garcinia cambogia; Hepatic collagen; Hepatic inflammation; Hepatic oxidative stress; Hydroxycitric acid; Metabolic syndrome; Tumor necrosis factor-α; Weight loss

Core tip: The preponderance of animal and human studies of Garcinia cambogia extract have found it to reduce markers of inflammation in brain, intestines, kidney and serum and to be either protective or neutral in terms of liver health. The limited reports of toxicities thus far have been linked to improperly manufactured materials and/or to peculiarities with the animal models used. The available data indicate that Garcinia cambogia extract/hydroxycitric acid does not cause liver toxicity.

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TO THE EDITOR

Kim *et al*[1] recently reported that a Garcinia cambogia extract (GC, 1%, w/w) fed to C57BL/6J mice in conjunction with a high-fat diet (HFD, 45 kcal% fat) for 16 wk protected against "HFD-induced obesity by modulating adipose fatty acid synthesis and β-oxidation but induces hepatic fibrosis, inflammation and oxidative stress[1]." A review of this article in light of other published research on (-)-hydroxycitric acid (HCA), the active component in GC, raises a number of questions. The most significant

are these: what was the form of HCA used (not indicated) and is the toxicity reported induced by HCA *per se* or, instead, was it caused by the source of HCA/GC tested? These issues are particularly acute inasmuch as the results of Kim *et al*[1] are at variance with numerous published studies involving both animals and humans, several of which indicate that HCA actually exerts a protective effect upon the liver.

Differences among various sources of HCA can be quite significant, yet against copious evidence, it too often is assumed that all sources of HCA are the same in terms of physiology. In the study in question, the tested compound is not fully identified. The only information provided is that the tested compound provided "1%, w/w, 60% hydroxyl citric acid" and was provided by Newtree Inc. of the United States (although Newtree appears to be a South Korean company). Whether this material was stabilized with calcium, potassium, sodium, *etc*. or some mixture of these is not revealed. Similarly, no information is provided on the free acid or lactone content, whether the extraction process is novel or established, the amount of residual toxins, such as chloride ion, left in the extract, and so forth and so on. For instance, some HCA calcium salts contain up to approximately six percent halogenated compounds due to improper processing of starting materials that had been dried with the help of sodium chloride-is the material used by Kim *et al*[1] one of these?

That the nature of the HCA-containing source is important was made clear years ago in a critical analysis of another study that purported to demonstrate toxicity, in that particular case, testicular toxicity, at high dosages. This was a study by Saito *et al*[2]. When examined closely by Burdock *et al*[3], it was determined that the HCA salt tested was very unusual in that it contained a high lactone content and that the weight loss results were not typical of literature on HCA. In this case, the particular animal model also turned out to have been inappropriately chosen. Hence, in an instance of supposed testicular toxicity, there were unacceptable levels of uncertainty about the compound being tested. Moreover, various aspects of the study design and its assumptions proved to be questionable.

Kim *et al*[1] remind the reader of the "potential for hepatotoxicity of hydroxycut, a formulation that contains GC among other ingredients[1]." Not mentioned is the fact that after almost two decades of free sale of HCA products, there appear to be no reports of human liver toxicity aside from those involving Hydroxycut and only 8 out of 14 of the Hydroxycut formulas associated with liver toxicity even contained HCA/GC! The safety, including liver safety, of HCA as relates to Hydroxycut and other products was evaluated at length by Stohs *et al*[4] and no evidence of toxicity was found. In retrospect, the common denominator in these cases appears to be green tea extracts. Animal models have established the hepatotoxicity of high oral doses of (-)-epigallocatechin-3-gallate[5]. Reviews of human usage strongly suggest a causal association, albeit an idiosyncratic one, between green tea consumption and liver damage[6]. In contrast, quite a number of reviews have affirmed that HCA is extremely safe. These include Chuah *et al*[7], Márquez *et al*[8]and Stohs *et al*[9].

Published studies involving both animal models and humans indicate that HCA *per se* is either neutral with regard to the liver or actively protective. For instance, GC in a rat model has been tested against toxic challenge to the liver by both ethanol and dexamethasone. Mahendran and Devi[10] demonstrated that GC supplementation was sufficient to prevent undesirable changes in the lipid profile on dexamethasone administration and also to protect normal liver phospholipid levels. Likewise, when rats were challenged with ethanol to induce peroxidation damage to the liver, Devi and Mahendran[11] found "co-treatment of the rats with Garcinia cambogia significantly inhibited the rise in lipid levels and also the peroxidative damage caused by ethanol, which is evident from the improved antioxidant status. The levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase were maintained at near normalcy in Garcinia cambogia treated rats". Similar protection was provided by GC with regard to liver superoxide dismutase, catalase and various glutathione compounds.

The study of Clouatre and Preuss, which lasted eight weeks and involved high-fat/high-sugar diets in rats, found results that are in line with those of Mahendran and Devi[10-12]. In healthy and relatively young animals, HCA treatment compared with control led to strong trends towards reduced CRP and tumor necrosis factor-α without exerting significant effects on ALT and AST. These results are similar to those found in a formal safety assessment of a commercial potassium-calcium hydroxycitrate salt (60% HCA) by Soni *et al*[13], which was designed, in part, specifically to look for potential hepatotoxicity. The gavage administration of this salt at doses up to 2500 mg/kg per day for a period of 90 d did not lead to any significant adverse effects, including in the histological examinations of the livers of the test and control arms. Given the high dosage of HCA and the time frame comparable to that of Kim *et al*[1], the results of the study by Soni *et al*[13] in the rat rodent model clearly are at variance with the findings in the mouse model and in line with other research reports of HCA's safety. The human equivalent dosage of HCA in the Soni study is approximately 30 g in a 60 kg individual.

There have been at least four published studies of the safety of HCA in humans and these trials reached the same conclusion confirming the safety of the oral consumption of HCA salts. The studies are those of Hayamizu *et al*[14], Hayamizu *et al*[15], Ishii *et al*[16] and Hayamizu *et al*[17]. None of these studies found any significant adverse effects on the liver. Hayamizu *et al*[14] in a study lasting three months, but involving only 1000 mg HCA per day (*i.e.,* 1666 mg of a 60% salt) found no significant change in any liver parameter. Hayamizu *et al*[16] found no observed adverse effects 4000 mg HCA per day for ten days and Hayamizu *et al*[17]. found no adverse effects at 3000 mg HCA for 30 d.

Finally, the issue of GC and inflammation needs to be

addressed more generally. Clouatre *et al*[18] were the first researchers to discover that HCA consumption relieves a number of markers of inflammation and this information was confirmed in Clouatre *et al*[12]. A number of recent studies now have established these findings regarding HCA and inflammation. A study using rats performed by dos Reis *et al*[19] found that the "antiinflammatory effects provided by the Garcinia cambogia extract result in an improvement of several parameters analysed (sic) in experimental colitis and could provide a source for the search for new antiinflammatory compounds useful in inflammatory bowel disease treatment." Similar protective effects have been found by Amin *et al*[20] in relation to high fat diet, metabolic disturbances and brain oxidative dysfunction and by Amin *et al*[21] in relation to renal oxidative stress on a high fat and high sucrose diet.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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